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(57) Abstract

Provided, among other things, is a method of preventing or ameliorating transplantation rejection reactions comprising treating the donor tissue with a rejection reaction preventing or ameliorating effective amount of a hydrolase that is effective to reduce the amount of one or more cell surface adhesion molecules.

papain p.32

p.37 (two step transplantation)

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TREATMENT AND PREVENTION OF IMMUNE REJECTION REACTIONS

The present application claims the priority of Provisional Patent Application No. 60/114,147, filed 24 December 1998.

The present invention relates to a treatments to prevent immune rejection reactions, such as graft vs. host disease (GVHD), with a hydrolase effective to remove cell surface adhesion molecules involved in triggering such immune reactions. One embodiment uses a krill-derived multifunctional enzyme and a family of crustacean and fish derived enzymes having substantial structural or functional similarity to the multifunctional enzyme derived from antarctic krill. Another particularly preferred enzyme is an Atlantic cod (*Gadus morhua*) trypsin, particularly that described *European J. Biochem.* 180: 85-94, 1989 and Protein Resource/GenBank Accession No. S03570.

The aquatic or other enzymes that are substantially or functional structurally similar to the krill-derived multifunctional enzyme have the same utility as the krill enzyme. In particular, these enzymes are useful for treating viral infections and other disorders, as outlined for example in U.S. Patent Application Nos. 08/486,820, 08/338,501 (filed November 22, 1994) and U.S. Patents 5,945,102 and 5,958,406.

A WO 96/00082 application of Cortecs Limited describes treating T-cells with bromelain to affect intracellular phosphorylation reactions. The mechanism is said to probably be blockage of "tyrosine phosphorylation of proteins including MAP kinase." The claims of the WO 96/00082 application recite preventing or treating tissue rejection, but no example or protocol for doing so is described. Using antibodies to CD3e and CD28, the authors concluded that "the removal of cell surface molecules by bromelain treatment was not responsible for the reduced cytokine mRNA observed," though *increases* in CD3e and CD28 binding observed "may have contributed" to a proliferative response observed.

A Döring et al., *J. Immunol.* 154: 4842-4850, 1995 article ("Döring") describes the effects of two enzymes found in sputum from cystic fibrosis patients on CD4 and CD8. The enzymes are polymorphonuclear leukocyte-derived proteinase elastase and cathepsin G. Exposure to the polymorphonuclear leukocyte-derived proteinase elastase apparently reduced the cytotoxic response of a T-cell clone. The authors speculate that the effect helps limit tissue damage from the sustained inflammation found in the lungs

of cystic fibrosis patients. Döring does not disclose any treatment of tissue rejection reactions.

Gaciong et al., *Transplantation Proceedings* 28: 3439-3440, 1996 assert that systemic administration of a mixture of bromelain, pancreatic trypsin and a glucoside reduces immune-mediated arteriosclerosis in rat model, where the rats received transplants of allogeneic abdominal aortas. Gaciong et al. describe no tissue-targeted method of preventing or ameliorating transplantation rejection.

SUMMARY OF THE INVENTION

The invention provides a method of preventing or ameliorating transplantation rejection reactions comprising treating the donor tissue with a rejection reaction preventing or ameliorating effective amount of a hydrolase (which can be a mixture of hydrolases), such as a protease. Without limiting the invention to theory, one indication that a hydrolase is appropriate for use in the invention is that such hydrolase is effective to reduce (e.g., remove, destroy, inactivate or disable) the amount of one or more cell surface adhesion molecules. For example, the invention can comprise selecting an hydrolase that is effective to induce tolerance in an immune cell to an antigen or cell to which the immune cell was previously reactive. Or, the invention can comprise selecting a hydrolase (or an appropriate mix of hydrolases) that disrupts signal 2 mediators of an immune cell or signal 1 mediators (or both). In one preferred embodiment, the invention can comprise selecting a hydrolase (or an appropriate mixture of hydrolases) that disrupts signal 2 mediators, while leaving in place signal 1. Alternatively, the invention can comprise selecting a hydrolase that removes, destroys, inactivates or disables at least 60% of the cell surface adhesion molecules involved in mediating signal 2 for which a purified krill-derived multifunctional enzyme ("PHM protease," as described, for example, in U.S. Patents 5,945,102 and 5,958,406) or cod trypsin removes, destroys, inactivates or disables at least 60%. Preferably, the amount of such cell surface adhesion molecules removed, destroyed, inactivated or disabled is an amount greater than or within 10% of the amount removed, destroyed, inactivated or disabled by PHM or cod trypsin. The method can comprise treating the donor tissue *ex vivo*.

In one embodiment, the hydrolase employed is more effective on a molar basis in preventing or ameliorating donor tissue rejection than is the krill multifunctional enzyme. For example, the hydrolase employed is more effective in removing one or more of CD4, CD8, CD25 (IL-2 alpha receptor chain), CD28, ICAM-1 (CD54), CD152

(also known as CTLA-4), GP39 (also known as CD154, CD40 ligand or CD40L), an integrin, CD40 and CD80 (also known as B7) than is the krill multifunctional enzyme. Or the hydrolase is more effective than papain, or bromelain, or mammalian trypsin. For example, the hydrolase employed is more effective in removing one or more of CD28, ICAM-1 (CD54), GP39 (CD154), an integrin, CD40 and CD80 than is the krill multifunctional enzyme. In another example, the hydrolase employed is more effective in removing CD28 than is one or more of the krill multifunctional enzyme. In another example, the hydrolase employed is more effective in removing ICAM-1 (CD54) than is one or more of the krill multifunctional enzyme. In another example, the hydrolase employed is more effective in removing an integrin than is one or more of the krill multifunctional enzyme. In another example, the hydrolase employed is more effective in removing an LFA-1 (also known as αL or CD11a) than is the krill multifunctional enzyme. In another example, the hydrolase employed is more effective in removing GP39 (CD154) than is one or more of the krill multifunctional enzyme.

In one embodiment of the method described above, the preventing or ameliorating transplantation rejection reactions comprises treating a donor source of immune cells (lymphocytes such as T-cells or B-cells) with a rejection preventing or ameliorating effective amount of a hydrolase that is effective to reduce the amount of one or more cell surface adhesion molecules, or preventing or ameliorating transplantation rejection. The method can comprise contacting the treated immune cells, which cells are obtained from a recipient animal, with second cells of a donor animal; and transplanting a tissue from the donor animal to the recipient animal

The invention also provides a method of preventing or ameliorating transplantation rejection reactions comprising: treating a donor source of immune cell (e.g., lymphocyte) *precursor* cells (such as from bone marrow) with a rejection preventing or ameliorating effective amount of a hydrolase, and administering the treated lymphocyte precursor cells to a recipient.

The invention further provides a method of preventing or ameliorating transplantation rejection reactions comprising: isolating from a source of immune cells taken from a donor (a) a fraction enriched in mature T-cells and (b) a fraction containing immune cell precursor cells; treating the mature T-cells of fraction (a) with a rejection preventing or ameliorating effective amount of a hydrolase; and administering the

mature T-cells of fraction (a) and fraction (b) to a recipient. In one embodiment, the hydrolase treated mature T-cells are contacted with cells of fraction (b) prior to administration to the recipient.

The invention still further provides a method of preventing or ameliorating transplantation rejection reactions comprising: treating a source of immune cells taken from a recipient or donor (for example where the recipient does not have an immune system) with a rejection preventing or ameliorating effective amount of a hydrolase; incubating the treated source of immune cells with a donor organ, tissue or cell type; transplanting the donor organ, tissue or cell type into the recipient; and administering the treated cells into the recipient. In one embodiment, the treated cells include mature T-cells.

The invention also provides a method of preventing or ameliorating allergic or autoimmune reactions comprising: treating a source of immune cells taken from a treatment subject or donor (for example where the recipient does not have an immune system) with an allergic or autoimmune reaction preventing or ameliorating effective amount of a hydrolase; exposing the immune cells to an antigen that induces the allergic reaction or which contains autoimmune epitopes; and restoring the treated and exposed immune cells cells to the treatment subject.

In one embodiment, the invention provides a method of preventing or ameliorating allergic, autoimmune or transplantation rejection reactions with a hydrolase, comprising: identifying the hydrolase or mixture of hydrolases as a hydrolase or mixture of hydrolases with a relative selective preference for disabling signal 2 and/or signal 1, or effective for inducing tolerance in immune cells to a substance or to a cell; treating immune cells with the hydrolase or mixture of hydrolases; and administering the treated cells to a mammal. Alternatively, the hydrolase can be selected on the basis of specificity for cell surface adhesion molecules.

The invention further provides (a) methods relating to certain conditions using effective amounts of hydrolase, (b) compositions for use in such methods, (c) pharmaceutical compositions containing effective amounts of hydrolase for use in such methods, and (d) uses of the hydrolase composition for manufacturing a medicament for use in such methods. The methods are include:

- treating a tissue, body fluid or composition of cells to remove or inactivate a cell adhesion component comprising, wherein the enzyme is administered to the tissue, body fluid or composition of cells, preferably a cell-adhesion component removing or inactivating effective amount or an immune rejection inhibiting amount of the enzyme is administered, wherein preferably the tissue, body fluid or composition of cells is treated extra-corporeally, although they may also be treated *in situ* in an animal; or
- treating or prophylactically preventing HIV infection, preferably administering an HIV infection treating or preventing effective amount of the enzyme

The method comprises administering a composition comprising a hydrolase described above.

The invention further provides (a) methods for treating or prophylactically preventing a cell-cell or cell-virus adhesion syndrome comprising administering an anti-adhesion effective amount of a hydrolase effective to remove or inactivate a cellular or viral acceptor or receptor adhesion component that is involved in the cell-cell or cell-virus adhesion, (b) compositions or substances for use in such methods, (c) pharmaceutical compositions containing effective amounts of enzyme for use in such methods, and (d) uses of the enzyme composition for manufacturing a medicament for use in such methods. Preferably, the syndrome comprises inflammation, shock, tumor metastases, autoimmune disease, transplantation rejection reactions or microbial infections. Preferably, (a) the syndrome is selected from the group consisting of graft versus host disease, organ or tissue transplantation rejection, autoimmune disease and associated conditions, microbial infection, immune disorder, cystic fibrosis, COPD, atherosclerosis, cancer, asthma, septic shock, toxic shock syndrome, conjunctivitis, reperfusion injury and pain, and (b) a cell surface adhesion molecule, associated with the cell-cell or cell-virus adhesion syndrome, is removed or inactivated by the administered hydrolase, where the cell surface adhesion molecule can be selected from the group consisting of ICAM-1 (also know as CD54), ICAM-2 (also known as CD102), VCAM-1, CD3, CD4, CD8, CD11, CD18, CD28, CD29D, CD31, CD44, CD 49, CD62L, CD102, GP39 (CD154), integrins (e.g., of β-1 subfamily {e.g., β-1 (CD29) with α1 (CD49a), α2 (CD49b), α3 (CD49c), α4 (CD49d), α5 (CD49e), α6 (CD49f) or αV (CD51), of β-2 subfamily (e.g., β-2 (CD11a) with αL (CD11b), αM (CD) or αX (CD11c), or of β-3

subfamily (e.g., β-3 (CD61) with αV (CD51) or α11b (CD41), β-4 (CD104) with α6 (CD49f), β-5 with αV (CD51), β-P with α4 (CD49d)} and asialo GM1 ceramide.

The invention further provides a pharmaceutical composition for removing or inactivating a cell-surface adhesion molecule comprising a cell-surface adhesion molecule removing or inactivating effective amount of a hydrolase. Such hydrolases include a number of enzymes such as cod trypsin and other hydrolases, including, as one specific example, proteases with multiple classes of proteolytic activity such as the multifunctional enzyme having: activity comprising at least one of a chymotrypsin, trypsin, collagenase, elastase or exo peptidase activity; a molecular weight between about 20 kd and about 40 kd as determined by SDS PAGE; and substantial homology to the krill-derived multifunctional hydrolase. Such compositions typically include a pharmaceutically acceptable diluent or carrier.

The invention still further provides a pharmaceutical composition for treating or prophylactically preventing a cell-cell or cell-virus adhesion syndrome comprising a cell-cell or cell-virus adhesion syndrome treating or preventing effective amount of a composition comprising a hydrolase. For example, in some embodiments the hydrolase is multifunctional enzyme having: activity comprising at least one of a chymotrypsin, trypsin, collagenase, elastase or exo peptidase activity; a molecular weight between about 20 kd and about 40 kd as determined by SDS PAGE; and substantial homology to the krill-derived multifunctional hydrolase. Such compositions typically include a pharmaceutically acceptable diluent or carrier.

In a preferred embodiment, HIV-infected patients are treated to slow the progression of the associated diseases by the process of (1) isolating T-cells from the patient, (2) treating the T-cells with a hydrolase effective to remove CD4, and (3) injecting the T-cells into the patient.

In one aspect, the method of extra-corporeally treating a tissue, body fluid or composition of cells to remove cell adhesion components reduces the immune rejection of a tissue, body fluid or composition of cells that is transplanted from one individual to another. In another aspect, such treatments remove or inactivate the cell adhesion components found in the treated tissue, body fluid or composition of cells involved in a microbial infection.

In some specific embodiments, the invention relates to a hydrolase having multifunctional activity comprising at least one of a chymotrypsin, trypsin, collagenase, elastase or exo peptidase activity, a molecular weight between about 20 kd and about 40 kd as determined by SDS PAGE, and substantial homology to krill-derived multifunctional hydrolase. Preferably, the enzyme has a molecular weight of from about 26 kd to about 32 kd as determined by SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis ("PAGE"), more preferably about 29 kd. Preferably, the enzyme has an N-terminal sequence comprising: I-V-G-G-X-E/D-B-X-X-X-X-Z/B′-P-Z/H-Q-B-X-B′/Z, wherein X is any amino acid, Z is an aromatic amino acid, B is an amino acid having a C1 to C6 alkyl side chain, and B′ is leucine or isoleucine. More preferably, all amino acids represented by X, Z or B are natural amino acids. Preferably, the enzyme has an N-terminal sequence comprising: I-V-G-G-X-E/D-B wherein X is any amino acid, B is an amino acid having a C1 to C6 alkyl side chain. Preferably, the enzyme is the krill-derived multifunctional hydrolase. Thus, in one embodiment, the N-terminal sequence is I-V-G-G-X-E-V-T-P-H-A-Y-P-W-Q-V-G-L-F-I-D-D-M-Y-F (SEQ ID NO. 20). Preferably, the enzyme has the N-terminal sequence: I-V-G-G-N/M-E-V-T-P-H-A-Y-P-W-Q-V-G-L-F-I-D-D-M-Y-F (SEQ ID NO. 1).

In these specific embodiments, preferably, the multifunctional enzyme of the invention has at least two of the identified proteolytic activities, more preferably at least three, still more preferably at least four. Yet more preferably, the enzyme has all of the identified proteolytic activities. Preferably, the multifunctional enzyme has substantial anti cell-cell and cell-virus adhesion activity. Preferably, the multifunctional enzyme has substantial homology with the krill-derived multifunctional hydrolase.

In another aspect of this specific embodiment, the multifunctional enzyme shall include an amino acid sequence having at least about 70% identity with a "reference sequence" described below, more preferably at least about 80% identity, still more preferably at least about 90% identity, yet still more preferably at least about 95% identity. The krill-derived multifunctional hydrolase can be the multifunctional enzyme. The reference sequence is (i) the amino acid 64-300 sequence of SEQ ID NO:21, or (i) a sequence which is that of the amino acid 64-300 sequence of SEQ ID NO:21 except that it has

one or more of the amino acid substitutions found in the amino acid 1-185 sequence of SEQ ID NO:22,

one or more of the amino acid substitutions found in the amino acid 72-178 sequence of SEQ ID NOS:23 or 24,

one or more of the amino acid substitutions found in the amino acid 1-211 sequence of SEQ ID NO:25,

one or more of the amino acid substitutions found in the amino acid 66-302 sequence of SEQ ID NO:26, or

has asparagine or lysine at a residue corresponding to residue 68 of SEQ ID NO:21,

wherein identity is calculated by (a) aligning the sequences as described below and determining, over the entire length corresponding to the reference sequence, the average number of substitutions, deletions or insertions for every 100 amino acids of the reference sequence, with this number corresponding to percent identity; or (b) the method of Needleman and Wunch, using the parameters set forth in Version 2 of DNASIS.

Preferably, the hydrolase is selectively reactive with cell-surface receptors such as proteins or glycolipids. Preferably, the hydrolase is substantially purified. In some embodiments, the hydrolase has a purity with respect to macromolecules of at least about 90%, more preferably least about 95%, more preferably about 97%, still more preferably about 99%, yet more preferably 99.7% with respect to macromolecules. For the purposes of this application, "substantially pure" shall mean about 60% purity.

The invention also provides a pharmaceutical composition comprising the multifunctional enzyme of claim 1 and a pharmaceutically acceptable diluent or carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B: Survival of (C57BL/6 x DBA/2)BDFI recipients of semi-allogeneic C57BL/6 bone marrow cells mixed with PHM-treated C57BL/6 splenocytes.

Figure 2: Survival of (C57BL/6 x DBA/2)BDFI recipients of semi-allogeneic C57BL/6 bone marrow cells mixed with protease-treated C57BL/6 splenocytes.

Figure 3 (3A-3C) shows the effects of cod trypsin of PHM incubations on surface markers.

Figures 4 and 5 show the effects of various hydrolases on a number of cell surface adhesion molecules.

DETAILED DESCRIPTION

It has now been established that the multifunctional enzyme and other hydrolases effectively remove or inactivate certain cell-surface adhesion molecules, such as ICAM-1 (CD54), ICAM-2 (CD102), VCAM-1, CD4, CD8, CD28, CD31, CD11a, CD49d, other integrin component chains, CD44, the asialo GM1 ceramide, CD40 and CD80 without affecting cell viability. This adhesion site removal or inactivation phenomenon is believed to provide at least a partial explanation for effectiveness against many, though probably not all, of the indications against which, for example, the multifunctional enzyme is effective.

Again not wishing to be limited by any particular theory, the anti-CD4 cell surface adhesion molecule activity of the multifunctional enzyme is believed to be responsible, at least in part, for the enzyme's HIV-transmission inhibitory activity. The HIV infective pathway utilizes the CD4 cell-surface molecule. *See*, Lentz, "Molecular Interaction of Viruses with Host-Cell Receptors," in *Adhesion Molecules*, Wegner, Ed., Academic Press, 1994, pp. 223-251 at p. 229.

Studies on the destruction or inactivation of cell surface molecules on T-cell exposed to as little as 10 μg/ml of the krill hydrolase for four hours at 37°C have determined that: CD3 and CD90 show little or no change; CD28, CD49, CD29D, CD18 and CD11 are significantly destroyed or inactivated, about 25% to about 40% reduction detectable antigen; ICAM-1 (CD54), ICAM-2 (CD102), CD44, CD31, CD62L (L-selectin), CD4, and CD8 are substantially destroyed or inactivated, generally about 70% to about 100% reduction in detectable antigen. Additionally, antibodies against asialo GM-1 have indicated reductions in the immunologically detectable amount of this ceramide in the membranes of lung epithelial cells following exposure to the multifunctional enzyme of the invention. Further, such treatment of lung epithelial cells with the krill hydrolase reduces the level attachment of *Pseudomonas* bacteria to the lung epithelial cells.

Further studies have established that hydrolases of interest remove certain cell surface molecules that are believed to contribute to the signal 2 pathway for activation of T-cells, these cell surface molecules include one or more of CD4, CD8, CD28, and CD154, while having substantially less effect on the T-cell receptor (TcR), which is involved in the signal 1 pathway for activation of T-cells. While not wishing to be

limited to theory, it is believed that hydrolases that substantially interfere with the signal 2 pathway or another accessory pathway (e.g., remove, destroy, inactivate or disable at least 60% of at least one CD4, CD8, CD28, or CD154) but do not substantially interfere with the signal 1 pathway (e.g., no more than 50% of TcR is removed, destroyed, inactivated or disabled) are effective in the immune rejection embodiments of the invention. It is believed that, for immune cells subjected to such differential disruption in pathways, exposure to certain substances initiates processes that lead to tolerance against immune reactions to those substances. *See*, e.g., Kuby, *Immunology*, Third Edition, W.H. Freeman & Co. 1997; Waldmann, "Transplantation Tolerance — Where Do We Stand," *Nature Med.* 5(11): 1245-1248, 1999. Prior work has indicated that intervention in signal 2, while signal 1 is activated, drives T-cells into an anergic state. The present inventors a shown that such selective disabling of signaling molecules can be done with hydrolases selected to have the appropriate selectivity in removing, destroying, inactivating or disabling cell surface molecules.

It is believed that the above discussed adhesion molecules and others will prove to play a role in a number of other diseases for which the multifunctional enzyme is an effective treatment or preventative agent. As described further in Example 4, it has now been shown that treatments with hydrolases are effective to treat, prevent or reduce the severity of GVHD.

For the purposes of this application, the terms listed below shall have the following meaning:

- **adhesion molecule:** a molecule found on the surface of a cell involved, directly or indirectly, in transmitting signals to the cell.
- **cell-cell or cell-virus adhesion syndrome:** a disease in which a receptor or acceptor cell adhesion component plays a role in the etiology of the disease, for instance by playing a role in the development, transmission, growth or course of the disease.
- **hydrolase:** an enzyme that degrades bonds formed by dehydration reactions such as amide, ester, or ether bonds, The term encompasses, but is not limited to, proteases such as trypsin and chymotrypsin.
- **identity:** "Identity," as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as the case may be, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence

relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" can be readily calculated by known methods, including but not limited to those described in (Computational Molecular Biology, Lesk, A.M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48: 1073 (1988). Preferred methods to determine identity are designed to give the largest match between the sequences tested. Thus, a contiguous portion of a polypeptide can be tested against the reference sequence described above and aligned to give the highest match taking into account that non-matched pairs and non-matched gap sequences are scored against identity, with the each non-matched pairing scoring and each non-matched gap residue or nucleotide reducing the identity, prior to normalization to a percent scale, by -1.

Thus, one of the simplest ways to describe polypeptide sequences that are related, as by high identity, is set forth below for a 95% identity example. In this case the test sequence includes a contiguous segment that is the reference amino acid sequence described above , or is identical with the reference sequence except that, over the entire length corresponding to the reference sequence, the amino acid sequence has an average of up to five substitutions, deletions or insertions for every 100 amino acids of the reference sequence.

Moreover, methods to determine identity are codified in publicly available computer programs. Computer program methods to determine identity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA (Altschul, S.F. et al., J. Molec. Biol. 215: 403-410 (1990). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, MD 20894; Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990). The method of Needleman and Wunch, using the parameters set forth in Version 2 of DNASIS can also be used. Additionally, the well known Smith Waterman algorithm can be used to determine identity.

Alternatively, Parameters for polypeptide sequence comparison include the following:

- Algorithm: Needleman and Wunsch, J. Mol Biol. 48: 443-453 (1970);
- Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff, Proc. Natl. Acad. Sci. USA. 89:10915-10919 (1992);
- Gap Penalty: 12; and
- Gap Length Penalty: 4

A program useful with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison WI. The aforementioned parameters are the default parameters for peptide comparisons (along with no penalty for end gaps).

By way of example, a polypeptide sequence of the present invention may be identical to the reference sequence, that is it may be 100% identical, or it may include up to a certain integer number of amino acid alterations as compared to the reference sequence such that the percent identity is less than 100% identity. Such alterations are selected from the group consisting of at least one amino acid deletion, substitution, including conservative and non-conservative substitution, or insertion, and wherein said alterations may occur at the amino- or carboxy-terminal positions of the reference polypeptide sequence or anywhere between those terminal positions, interspersed either individually among the amino acids in the reference sequence or in one or more contiguous groups within the reference sequence.

• **immune disorder:** any disorder caused by an immune reaction to foreign substances, tissues or cells or to autologous or transplanted tissue. The term encompasses autoimmune diseases.

• **immune cell:** a lymphocyte, such as a B-cell or T-cell, or a precursor cell to a lymphocyte.

• **krill-derived multifunctional hydrolase:** a multifunctional enzyme having the same sequence as the enzyme isolated from krill having the properties of the protein described in Examples 1B, 1C and 1D. This enzyme is also referred to as the "krill multifunctional hydrolase" or the "krill multifunctional enzyme" or the "krill-derived multifunctional enzyme."

• **macromolecule:** for determining purity, this means a biological polymer such as a protein, nucleic acid or carbohydrate of molecular weight greater than about 1000.

- **multifunctional enzyme:** an enzyme having activity comprising at least one of a chymotrypsin, trypsin, collagenase, elastase or exo peptidase activity, a molecular weight between about 20 kd and about 40 kd, and substantial homology to krill-derived multifunctional hydrolase.
- **multifunctional enzyme derived from fish or crustacean:** refers to an enzyme having the same sequence as an enzyme isolated from fish or crustacean.
- **protein:** for the purpose of determining purity, this means a polypeptide of molecular weight greater than about 1000.
- **reactive with a cell-surface protein or glycolipid:** means removes, destroys, inactivates or disables the detectable presence of the cell-surface molecule, by whatever mechanism.
- **reactive with a cellular or viral acceptor or receptor adhesion component:** means removes, destroys, inactivates or disables a cell's or a virus' ability to interact with a cell, virus, ligand, group or molecule, regardless of the mechanism.
- **SDS-PAGE:** means polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulfate.
- **selectively reactive with a cell-surface protein:** means removes, destroys, inactivates or disables certain cell-surface proteins on the surface of a cell but not others.
- **substantial homology:** at least about 60% sequence homology.
- **systemic administration:** an administration of a biological agent, such as the multifunctional enzyme, designed deliver the agent to the blood or other circulatory system (such as the lymphatic system) of an animal.
- **tolerance:** a state of unresponsiveness of an immune cell upon encountering an antigen or cell.
- **units of activity:** Hydrolases have unit activity according to a recognized assay for the particular type of hydrolase, and is typically defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of substrate per minute at 25°C. For the chymotrypsin activity of a hydrolase, succinyl-ala-ala-pro-phe-*p*-nitroanilide (Sigma Chemical Co., St. Louis, MO) is the substrate, and hydrolysis is monitored via the absorbance change at 410 nm. The extinction coefficient, ε, of *p*-nitroanilide is 8800 $M^{-1}cm^{-1}$, thus the multiplication factor to convert dA/minute into U/minute of sample is

5.68, when 20 μl of sample is used. For the trypsin activity of a hydrolase, the substrate is CBZ-GRPpNA.

When HL60 cells (promyelocyte cells believed to give rise to T-cells, derived from an acute progranulocytic leukemia) are pretreated with the krill multifunctional hydrolase, their binding to TNFα stimulated endothelial cells is inhibited by more than about 60%. Preferably, treatment of HL60 or endothelial cells with the multifunctional enzyme of the invention will inhibit HL60 cell binding to TNFα stimulated endothelial cells by at least about 20%, more preferably at least about 40%, still more preferably at least about 60%, yet more preferably at least about 80%. Alternately, the multifunctional enzyme will preferably have at least about 30% of the adhesion-inhibiting activity of the krill-derived multifunctional hydrolase. More preferably, the multifunctional enzyme shall have at least about 60% of the adhesion inhibiting activity of the krill-derived multifunctional hydrolase, still more preferably at least about 80%, yet more preferably at least about 100%.

Transplantation; Autoimmune and Allergic Reactions

Studies on bone marrow transplantation provide an illustration of the effect of hydrolase treatments in treating, inhibiting or preventing an immune rejection, in this case GVHD. GVHD typically involves the donor cells attacking the host, instead of the host attacking the donor tissue. Bone marrow transplantation (BMT) is used in conjunction with treatments of a number of cancers, particularly treatments that damage or destroy cell types found in blood, such as treatments of life-threatening hematologic malignancies. However, the threat of severe graft-vs.-host disease (GVHD) remains a major obstacle, impeding widespread application of bone marrow transplantation. Acute and chronic GVHD develops in a significant proportion of transplant recipients and represents a major cause of morbidity and mortality after bone marrow transplantation between imperfectly matched individuals (i.e., allogeneic transplantation). Efforts to prevent GVHD should reduce morbidity and mortality of transplantation, and enhance the long term outcome of a transplant. GVHD is a T-cell mediated disease affecting multiple organ systems. The risk of death due to GVHD can be reduced by depleting the T-cell population in the marrow inoculum used in bone marrow transplantation, or by using immunosuppressive drugs, such as FK506 or rapamycin (see for review, Blazar et al., 1997). Others have shown that a short course of high dose IL-2 administered at the

time of bone marrow transplantation can protect against GVHD mortality in mice (Sykes et al., 1990; Abraham et al,, 1992). Treatment with a protective course of IL-12 also inhibit GVHD, as IL-12 reduces the kinetics of T-cell expansion (Sykes et al., 1995). In most of these strategies, however, extensive treatments ancillary to transplantation are necessary, and can lead to adverse consequences.

Recent strategies against GVHD have evolved around the concept of inducing immune tolerance in T-cells. In the late 1980's, Jenkins and Schwartz demonstrated that to get full activation, T-cells must receive two signals: one through the T cell receptor (TcR), and a second signal delivered by accessory molecules, such as CD28, which bind to their counter receptors expressed at the surface of antigen presenting cells (APC) (reviewed in Schwartz et al., 1997). Activation of T cells through the TcR in absence of the second signal not only fails to activate T cells, but to the contrary induces a state of unresponsiveness (i.e., anergy). Close interactions between cells also play a crucial role in allorecognition as such interactions facilitate the binding of the TcR to the allo-MHC, and of the accessory receptor to its counter ligand. Indeed, integrins like LFA-1 (αL or CD11a, which associates with CD18) expressed on T cells bind to a counter ligand (ICAM-1, i.e., CD54) on the antigen presenting cell to increase the avidity of the interaction between a T-cell the antigen presenting cell (Dustin et al., 1991; St-Pierre et al., 1991). Consistent with this model is the observation that blocking LFA-1/ICAM-1 interactions with antibodies prevents GVHD only partially, but such blockade significantly increases the efficacy of other blocking antibodies specific for other accessory molecules in inducing a state of anergy in T-cells during GVHD (Blazar et al., 1995; Cavazzana-Calvo et al., 1996).

Treatment of immune cells with hydrolases significantly affects key cell surface adhesion molecules implicated in the delivery of activation signals. It has now been found that CD4, CD8, and other cell adhesion molecules, are among the most sensitive cell surface adhesion molecules to proteolysis. *Ex vivo* treatment of donor T-cells with hydrolase prior to engraftment is believed to block these activation signals and significantly reduce the severity of GVHD. The present work reports the results of two series of experiments in which lethal GVHD was prevented by treatment of mature T-cells with hydrolase (*see*, Example 4). In one experiment, both the krill multifunctional enzyme and a Cod-derived trypsin were effective. In the other experiment, the Cod

trypsin was more effective, probably reflecting the faster digestion kinetics observed with this enzyme.

It is important to note that the protection induced by *ex vivo* treatment of splenocytes with hydrolases was obtained by treating spleen cells of the donor. Hydrolase treatment of spleen cells is believed (without limitation to theory) to prevent full activation of allogeneic T-cells, inducing a state of tolerance that is transferred to bone marrow T-cells and their precursors through the immune mechanism known as "infectious tolerance" (Cobbold and Waldmann, 1998). Thus, the results reported herein have significant impact not only in GVHD resulting from bone marrow transplant, but on solid organ transplantation as well. *Ex vivo* treatment of recipients T-cells with hydrolase, followed by exposure to allogeneic donor MHC, is believed to induce a state of tolerance in these T-cells that is propagated systemically upon re-injection into the recipient. In some embodiments, exposure to donor MHC is conducted *in vitro* (i.e., also *ex. vivo*).

Without limitation to theory, it is believed that the transplantation rejection inhibition seen with the present invention can be explained if the hydrolase-treated immune-mediating cells, when brought into contact with the cells or substances which would trigger immune responses, instead begin the process of acquiring tolerance for such cells or substances. When treated cells are reintroduced into a recipient, such acquired tolerance is believed to be transmitted to other immune cells.

When immune cells are treated and contacted with other immune reaction mediating cells prior to administration to a patient, such contacting is, for example, conducted under appropriate conditions for maintaining metabolically active immune reaction mediating cells for, for example, from a few minutes to a few hours, preferably from about 1 hour to about 4 hours.

In the invention, immune reaction mediating cells are treated with hydrolase, exposed to a preparation which would trigger the immune response sought to be avoided, and reintroduced into a treatment subject. Such hydrolase treatment is typically *ex vivo*, and the exposure is preferably conducted *ex vivo*. Such *ex vivo* exposing (i.e., contacting) is, for example, conducted under appropriate conditions for maintaining metabolically active immune reaction mediating cells for, for example, from a few minutes to a few hours, preferably from about 1 hour to about 4 hours.

Examples of autoimmune-associated antigen preparations include, without limitation, myelin sheath preparations, myelin basic protein and preparations of one or more types of collagen. Antigen preparations can be used, for example, in the treatment of multiple sclerosis, irritable bowel disease (including Crohn's Disease and ulcerative colitis), pernicious anemia, juvenile onset diabetes, thyroiditis, systemic lupus erythematosis (SLE), scleroderma, polyarteritis nodosa and other vasculitides, myasthenia gravis, motor neuron disease, encephlomyelitis, subacute sclerosing pan-encephalitis, Goodpasture's Syndrome, haemolytic anemia, thrombocytopenia, pemphigus vulgaris and bullous pemphigoid. Other examples of autoimmune diseases and examples of allergies can be found in standard texts on allergies or immunology, such as Roitt, *Essential Immunology*, Eighth Edition, Blackwell Scientific Publications, Oxford, 1994.

Transplantation or tolerizing protocols according to the invention include:

1.	Contacting first cells which are immune cells from a recipient animal with second cells or immunogens to which one seeks to induce tolerance, where the first cells or the second cells are immune cells that are treated with hydrolase, administering the first cells to the recipient animal, and, if appropriate, transplanting tissue from the animal source of the second cells to the recipient; and
2.	Contacting first cells which are immune cells from a donor animal with second cells, wherein the first cells or second cells comprise antigens to which one seeks to induce tolerance, where the first cells or the second cells are immune cells that are treated with hydrolase, administering the first cells to a recipient animal, and, if appropriate, transplanting tissue form the animal source of the second cells to the recipient.

The cells contacted with the treated immune cells can be the tissue to be transplanted. Typically, the immune cells are administered some time before transplantation, such as 12, 24, 48, 72 hours. The second cells can also be treated with the hydrolase.

Preferably, the tolerized cells are T-cells. Thus, in one embodiment, the T-cells are tolerized by contact with T-cell depleted cells, preferably immune cells. The T-cells can then be isolated from the tolerizing mixture of cells by an affinity binding protocol or

cell sorting with appropriate cell-specific antibody reagents. For example, Thy-1 (CD90) antibodies tagged with a magnetically susceptible material can be used to isolate T-cells by by magnetic separation.

Exemplary Hydrolases

A wide variety of hydrolases are believed to be applicable. These include metalloproteinases (such as matrix metalloproteinases, including human fibroblast collagenase, interstitial collagenase, stromelysin, gelatinase A, gelatinase B, adamalysins, microbial metalloproteinases and the like), elastases, trypsins, chymotrypsins, other serine proteinases, and the like. Such hydrolases include hydrolases of aquatic origin, as described herein. Other applicable hydrolases are believed to include, for example, mammalian and non-mammalian trypsins, mammalian and non-mammalian chymotrypsins, mammalian and non-mammalian elastases, papains, bromelains, mammalian and non-mammalian collagenases, subtilisins and mammalian and non-mammalian cathepsins (such as cathepsin B, C, D or G). Further enzymes include mixtures of digestive enzymes from Atlantic cod (e.g., trypsin, chymotrypsin, elastase and collagenase), chymotrypsins for Atlantic cod (see, Aseirsson and Bjarnason, *Comp. Biochem. Physiol.* 99B:327-335, 1991; Guthmundsdottir et al., *Biochem. Biophys. Acta.* 1219:211-214, 1994), elastase from Atlantic cod (Aseirsson and Bjarnason, *Biochem. Biophys. Acta.* 1164:91-100, 1993), a mixture of serine proteinase-type collagenases from Atlantic cod (see, Kristjansson et al., *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 110:707-717, 1995), trypsin from Atlantic cod (Aseirsson et al., *Eur. J. Biochem.* 180:85-94, 1989), collagenase from *Uca pugilator* (Tsu et al., *J. Biol. Chem.* 269:19565-19572, 1994), and other hydrolases described herein.

In certain embodiments of the invention, the hydrolase used is not, at least in effective amounts, bromelain, or, in some embodiments, an enzyme component of bromelain. Bromelain is an enzyme-containing extract from pineapple, particularly pineapple stem. Also in certain embodiments, the hydrolase used is not, at least in effective amounts, polymorphonuclear leukocyte-derived proteinase elastase or cathepsin G. Further, in certain embodiments the hydrolase used is not, at least in effective amounts, papain, or mammalian pancreatic trypsin, or a mixture of these enzymes. Papain is an enzyme extracted from papaya.

Signal 2 and Signal 1 Pathways

Preferably, a hydrolase exposed to immune cells removes, destroys, inactivates or disables at least 60%, 75%, 85%, 90%, 95%, 98% or 99% of at least one of CD4, CD8, a CD11, CD25, CD28, a CD49, CD152 and CD154. These molecules are involved in the signal 2 pathway. Preferably, the hydrolases removes, destroys, inactivates or disables a significant portion of (a) CD4 and CD8, (b) CD4 and CD28, (c) CD4 and CD154, (d) CD4, CD8 and CD28, (e) CD4, CD8 and CD154, (f) CD4, CD28 and CD154, (g) CD4, CD8, CD28 and CD154, (h) CD8 and CD28, (i) CD8 and CD154, (j) CD8, CD28 and CD154, or (k) CD28 and CD154. In certain preferred embodiments, the hydrolases removes, destroys, inactivates or disables a significant portion of (a) CD4 and CD8, (d) CD4, CD8 and CD28, (e) CD4, CD8 and CD154 or (g) CD4, CD8, CD28 and CD154.

Preferably, no more than 50%, 35%, 20% or 10% of TcR, which is associated with signal 1, is removed, destroyed, inactivated or disabled by contacting a hydrolase with the immune cells. Preferably, no more than 50%, 35%, 20% or 10% of CD3, which is associated with signal 1, is removed, destroyed, inactivated or disabled by contacting a hydrolase with the immune cells.

Mixtures of Hydrolases

In certain embodiments, a mixture of two or more hydrolases is used to provide the removing, destroying, inactivating or disabling activity, i.e., induce tolerance, or the signal 2 disrupting activity. The mixture can be selected on the basis of mixing a second (or third, etc.) hydrolase that is more effective against given cell surface adhesion molecule than another hydrolase in the mixture.

Co-Administration of Antibodies

In one embodiment of the invention, the effects of hydrolase treatment is supplemented with the use of antibodies to specific cell adhesion molecules. This approach can be used for example to alter the kinetics of cell surface effects or supplement effects against certain cell adhesion molecules. For example, the hydrolase selected could be very effective against certain of the targeted cell surface adhesion molecules, but less effective against others. In this case, the antibodies, which are preferably monoclonal, are used to target the cell surface adhesion molecules against which the hydrolase is less effective. Or, the selected hydrolase can be effective against targeted cell surface adhesion molecules, but a further effect can be achieved with the antibodies. The antibodies can monovalent (e.g., fab fragments), especially as to a given

cell surface target (such that an antibody monovalent as to a cell surface adhesion molecule has another binding pocket with another specificity).

Appropriate targets for antibody blockade include CD4, CD8, CD25 (IL-2 receptor alpha chain), CD28, CD152 (CTLA-4), integrins, CD154, CD40 and CD80.

Antibody sources for use in this aspect of the invention include: Boehringer Mannheim, LaVal, Quebec; GIBCO, New York; PharMingen, San Diego, CA; Wako Bioproducts, Richmond, VA. The antibodies are contacted with the cells in sufficient amounts, and preferably some excess, to bind the available targeted cell surface adhesion molecules.

Administration of Hydrolase

For topical treatments, a suitable dose of hydrolase per application ranges from about 0.1 μg/cm^2 to about 1 mg/cm^2, preferably from about 1 μg/cm^2 (for example, using about 10 μg/ml) to about 1 mg/cm^2 (for example, using about 10 mg/ml), more preferably from about 5 μg/cm^2 (for example, using about 50 μg/ml) to about 100 μg/cm^2 (for example, using about 1 mg/ml), yet more preferably from about 10 μg/cm^2 to about 250 μg/cm^2, still yet more preferably from about 10 μg/cm^2 (for example, using about 100 μg/ml) to about 50 μg/cm^2 (for example, about 500 μg/ml). For systemic treatments, dosages will generally be selected to maintain a serum level of hydrolase between about 0.1 μg/100cc and about 5 μg/100cc, preferably between about 0.5 μg/100cc and about 2.0 μg/100cc. In an alternative measure of preferred systemic administration amounts, preferably from about 0.1 mg/kg to about 10 mg/kg, more preferably about 1mg/kg, will be administered (although toxicology in animal models suggests that amounts even in excess of 25 mg/kg can be used). For ocular treatments, a suitable dose of hydrolase per application ranges from about 0.01 mg per eye to about 5 mg per eye, preferably from about 0.1 mg per eye to about 2.0 mg per eye. For vaginal and urinary tract treatments, suitable flushing/ instillation solutions of the hydrolase will generally have concentrations from about 1 μg/ml to about 15 mg/ml, preferably from about 100 μg/ml to about 3 mg/ml. For oral treatments, suitable mouthwash solutions will generally have concentration of hydrolase from about 1 mg/ml to about 15 mg/ml preferably from about 2 mg/ml to about 10 mg/ml. Lozenges will typically contain from about 100 μg to about 10 mg of hydrolase. Aerosols will generally be made from solutions having enzyme concentrations from about 0.1 mg/ml to about 15 mg/ml,

preferably from about 1 mg/ml to about 10 mg/ml. Generally, from about 0.1 ml to about 2 ml of aerosol will be applied to the airways of the patient, preferably from about 0.5 ml to about 1.0 ml. For scar and keloid treatments, generally between about 0.1 mg and about 5 mg of hydrolase will be injected into each cm^2 of the lesion, preferably from about 0.5 mg to about 3 mg. For treating adhered connective tissue or joints, generally between about 0.5 mg and about 10 mg of hydrolase will be injected interstitially at the adhesion, preferably between about 1 mg and about 5 mg. For all treatments, the enzyme composition will generally be applied from about 1 to about 10 times per day, preferably from about 2 to about 5 times per day. These values, of course, will vary with a number of factors including the type and severity of the disease, and the age, weight and medical condition of the patient, as will be recognized by those of ordinary skill in the medical arts. It is believed that substantially higher doses can be used without substantial adverse effect.

For treating immune disorders, the composition may be applied systemically or in a manner adapted to target the affected tissue or cells, or a tissue or cells implicated in the disorder can be treated extra-corporeally.

For organ transplants or other *ex vivo* treatments, the organ, tissue or cells to be transplanted will preferably be bathed in a solution of the hydrolase for between about 10 minutes and about 5 hours. The enzyme solution will preferably contain between about 0.01 mg/ml or 0.5U/ml and about 25 mg/ml or 1,250U/ml of the hydrolase, and in certain embodiment preferably, between about 0.5 mg/ml or 25U/ml and about 5 mg/ml and about 250U/ml. After transplantation, the hydrolase can be administered systemically using the conditions described above. For treating bone marrow or other sources of cells found in the blood, particularly those containing T-cells or T-cell precursors, the cells are preferably treated with an amount and time of treatment effective to reduce, remove or inactivate at least one cell surface protein by at least about 50%, more preferably by at least about 80%.

The hydrolase of the invention is administered orally, topically, rectally, vaginally, by instillation (for instance into the urinary tract or into fistulas), by pulmonary route by use of an aerosol, by application of drops to the eye, or systemically, such as parenterally, including, for example, intramuscularly, subcutaneously, intraperitoneally, intraarterially or intravenously. The multifunctional enzyme is

administered alone, or it is combined with a pharmaceutically-acceptable carrier or excipient according to standard pharmaceutical practice. For the oral mode of administration, the hydrolase is used in the form of tablets, capsules, lozenges, chewing gum, troches, powders, syrups, elixirs, aqueous solutions and suspensions, and the like. In the case of tablets, carriers that is used include lactose, sodium citrate and salts of phosphoric acid. Various disintegrants such as starch, and lubricating agents such as magnesium stearate and talc, are commonly used in tablets. For oral administration in capsule form, useful diluents are lactose and high molecular weight polyethylene glycols. If desired, certain sweetening and/or flavoring agents are added. For parenteral administration, sterile solutions of the hydrolase are usually prepared, and the pHs of the solutions are suitably adjusted and buffered. For intravenous use, the total concentration of solutes should be controlled to render the preparation isotonic. For ocular administration, ointments or droppable liquids may be delivered by ocular delivery systems known to the art such as applicators or eye droppers. Such compositions can include mucomimetics such as hyaluronic acid, chondroitin sulfate, hydroxypropyl methylcellulose or polyvinyl alcohol, preservatives such as sorbic acid, EDTA or benzylchronium chloride, and the usual quantities of diluents and/or carriers. For pulmonary administration, diluents and/or carriers will be selected to be appropriate to allow the formation of an aerosol. For topical administrations, the hydrolase is typically administered in aqueous form or in a hydrogel. A preferred hydrogel comprises an aqueous suspension of from about 1% (w/v) to about 10% of low molecular weight hydrolyzed starch.

Suppository forms of the hydrolase are useful for vaginal, urethral and rectal administrations. Such suppositories will generally be constructed of a mixture of substances that is solid at room temperature but melts at body temperature. The substances commonly used to create such vehicles include theobroma oil, glycerinated gelatin, hydrogenated vegetable oils, mixtures of polyethylene glycols of various molecular weighty and fatty acid esters of polyethylene glycol. *See*, Remington's Pharmaceutical Sciences, 16th Ed., Mack Publishing, Easton, PA, 1980, pp. 1530-1533 for further discussion of suppository dosage forms. Analogous gels or cremes can be used for vaginal, urethral and rectal administrations.

Numerous administration vehicles will be apparent to those of ordinary skill in the art, including without limitation slow release formulations, liposomal formulations and polymeric matrices.

For adhesion disorders, the cells or viruses involved can include, without limitation, endothelial cells, lymphocytes, including T-cells, tumor cells, microbial cells, viruses, including HIV and herpes. Adhesion processes are believed to be involved in tissue invasion, for instance, by immune cells, microbes, and tumor cells.

Preferred hydrolases are proteases. Particularly preferred is the multifunctional enzyme of the invention.

Generally, the hydrolase will be administered in an effective amount. An effective amount is an amount effective to either (1) reduce the symptoms of the disease sought to be treated, (2) induce a pharmacological change relevant to treating the disease sought to be treated, (3) inhibit or prevent infection or re-infection by an infective agent, or (4) prevent or minimize the occurrence of a non-infectious disease (for instance a disease treatable by blocking a cell adhesion phenomenon).

Humans are the preferred subjects for treatment. However, the hydrolases can be used in many veterinary contexts to treat animals, preferably to treat mammals, as will be recognized by those of ordinary skill in light of the present disclosure.

The adhesion of HL60 cells (a hyman lymphocyte cell line) to endothelial cells is believed to model a mechanism for tumor cell invasion and infection more generally. This adhesion is stimulated by tumor necrosis factor ("TNF ") and inhibited by antibodies to the E-selectin antigen on HL60 cells. E-selectin is a cell surface adhesion protein that appears to bind to a sialated carbohydrate. See, Bevilacqua et al., *Science* (1989) 243:1160.

Preparations of the multifunctional enzyme are active even when not purified to homogeneity. Preparations are described, for example, in WO 96/24371 (Phairson Medical) and WO 98/08863 (Phairson Medical).

Isolations and partial sequences of various fish or crustacean hydrolases have been reported. A number of such reports are identified in Table 1, below.

Table 1 - Sequence Reports

Penaeus vanamelii 1		
	Sequence reported:	Van Wormoudt et al., *Comp Biochem. Physiol.*, 103B: 675-680, 1992 and Sellos and Wormhoudt, *FEBS*, 39: 219-224, 1992.
	Reported activities:	chymotryptic
	Apparent MW:	25kd
Panaeus vanameii 2		
	Sequence reported:	Van Wormoudt et al., *Comp Biochem. Physiol.*, 103B: 675-680, 1992.
	Reported activities	chymotryptic (tryptic)
	Apparent MW:	25kd
Panaeus monodon tryptic (shrimp)		
	Sequence reported:	Lu et al., *Biol. Chem. Hoppe-Seyler*, 371: 851-859, 1990.
	Reported activities:	tryptic
	Apparent MW:	27kd
	Ph optimum:	7.4 - 8.0
	Pi:	2.4
Panaeus monodon chymotryptic - 1 (shrimp)		
	Sequence reported:	Tsai et al., *Biochem et Biophys. Acta*, 1080: 59-67, 1991
	Reported activities:	chymotryptic collagenase
	Apparent MW:	27-28kd
Panaeus monodon chymotryptic - 2		
	Sequence reported:	Tsai et al., *Biochem. et Biophys. Acta*, 1080: 59-67, 1991
	Reported activities:	chymotryptic collagenase
	Apparent MW:	25-26kd
Uca pubilator (Fiddler Crab) 1		
	Sequence reported:	Tsai et al., *Biochem. et Biophys. Acta*, 1080: 59-67, 1991
	Reported activities:	chymotryptic
	Apparent MW: Ph optimum	25kd 8.0 - 8.5

Uca pugilator II		
	Sequence reported:	Grant et al., *Biochemistry*, 19: 4653-4659, 1980.
	Reported activities:	chymotryptic collagenase tryptic elastase
	Apparent MW:	25kd
	pI:	8.0 - 8.5
Kamchatka crab (at least four proteases)		
	Sequence Reported:	Klimova et al., *Biochem. Biophys. Res. Commun.* **166**: 1411-1420, 1990
	Reported Activities:	tryptic collagenase
	Apparent MW:	23-26kd
Crayfish Protease		
	Sequence reported:	Titani et al., *Biochemistry*, 22: 1459-1465,

The sequence of the first 25 amino acids of the Krill derived multifunctional enzyme is I-V-G-G-N/M-E-V-T-P-H-A-Y-P-(W)-Q-V-G-L-F-I-D-D-M-Y-F (SEQ ID NO. 1). The parentheses indicate a weak recovery of the 14th amino acid and "N/M" indicates heterogeneity at the 5th position. A comparison of the N-terminal 20 to 25 amino acid sequences of various serine hydrolases is presented in Table 2, below.

Table 2 - N-Terminal Sequences

Species	SEQ ID NO	Sequence
Penaeus vanameii 1 (shrimp)	3	I V G G V E A T P H S W P H Q A A L F I D D M Y F
Penaeus vanameii 2	4	I V G G V E A T P H S X P H Q A A L F I
P. monodon, trypt. (shrimp)	5	I V G G T A V T P G E F P Y Q L S F Q D S I E G V
P. monodon, chym. 1	6	I V G G V E A V P G V W P Y Q A A L F I I D M Y F
P. monodon, chym. 2	7	I V G G V E A V P H S W P Y Q A A L F I I D M Y F
Uca pugilator I (crab)	8	I V G G V E A V P N S W P H Q A A L F I D D M Y F

Species	SEQ ID NO	Sequence
Uca pugilator II	9	I V G G Q D A T P G Q F P Y Q L S F Q D
King crab	10	I V G G Q E A S P G S W P ? Q V G L F
Kamchatka crab I	11	I V G G Q E A S P G S W P X Q V G L F F
IIA	12	I V G G T E V T P G E I P Y Q L S L Q D
IIB	13	I V G G T E V T P G E I P Y Q L S F Q D
IIC	14	I V G G S E A T S G Q F P Y Q X S F Q D
Crayfish	15	I V G G T D A T L G E F P Y Q L S F Q N
krill Enzyme	1	I V G G N E V T P H A Y P W Q V G L F I D D M Y F
	2	I V G G M E V T P H A Y P W Q V G L F I D D M Y F
Bovine chymotrypsn	16	I V N G E D A V P G S W P W Q V S L Q D
Salmon	17	I V G G Y E C K A Y S Q A Y Q V S L N S G Y H Y C
Atlant. Cod I*	18	I V G G Y E C T K H S Q A H Q V S L N S G Y H
Atlant. Cod II*	19	I V G G Y E C T R H S Q A H Q V S L N S G Y H
Atlant. Cod Tryspin	27	I V G G Y Q C E A H S Q A H Q V S L N S G Y H Y C G G S L I N W V V S A A

*Both of these enzymes are trypsins; see, Gudmundsdottir et al., *Eur. J. Biochem.* **217**: 1091-1097, 1993.
X = unknown or undefined.

It will be apparent to those of ordinary skill that the enzyme can be manufactured by recombinant means. For instance, the sequences recited herein can be used as the basis of oligonucleotide probes for screening expression or genomic libraries to isolate the complete structural gene. *See, e.g.*, Suggs et al., *Proc. Natl. Acad. Sci. USA*, 78: 6613, 1981 or Berent et al., *BioTechniques*, 3: 208, 1985. Alternately, known protein sequences can be used to design primers for use in PCR-based amplification of nucleic acid encoding a multifunctional enzyme. *See generally, Molecular Cloning: A Laboratory Manual, second edition*, Cold Spring Harbor, 1989 and *PCR Protocols, A Guide to Methods and Applications*, edited by Michael et al., Academic Press, 1990. Once fully identified, these structural genes can be edited and appropriately inserted into expression vectors by methods known to the art. In particular, recombinant means can follow the guidance found in WO 98/08863 (Phairson Medical).

These structural genes can be altered by mutagenesis methods such as that described by Adelman et al., *DNA*, 2: 183, 1983 or through the use of synthetic nucleic acid strands. The products of mutant genes can be readily tested for multifunctional enzymatic activity. Conservative mutations are preferred. Such conservative mutations include mutations that switch one amino acid for another within one of the following groups:

1. Small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro and Gly;
2. Polar, negatively charged residues and their amides: Asp, Asn, Glu and Gln;
3. Polar, positively charged residues: His, Arg and Lys;
4. Large aliphatic, nonpolar residues: Met, Leu, Ile, Val and Cys; and
5. Aromatic residues: Phe, Tyr and Trp.

A preferred listing of conservative substitutions is the following:

Original Residue	**Substitution**
Ala	Gly, Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Ala, Pro
His	Asn, Gln
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Tyr, Ile
Phe	Met, Leu, Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp, Phe
Val	Ile, Leu

The types of substitutions selected can be based on the analysis of the frequencies of amino acid substitutions between homologous proteins of different species developed by Schulz et al., *Principles of Protein Structure*, Springer-Verlag, 1978, pp. 14-16, on the analyses of structure-forming potentials developed by Chou and Fasman, *Biochemistry*

13, 211, 1974 or other such methods reviewed by Schulz et al, *Principles in Protein Structure*, Springer-Verlag, 1978, pp. 108-130, and on the analysis of hydrophobicity patterns in proteins developed by Kyte and Doolittle, *J. Mol. Biol.* 157: 105-132, 1982.

Krill, including without limitation krill of the genuses *Euphausia* (such as superba, crystallorphias, frigida, triacantha, vellantini, lougirostris, lucens, similis, spinifera, recurva and the like), *Meganyctiphanes* (such as norvegica and the like) and *Tysanoessa* (such as macurura, vicina, gregaria and the like), are a preferred source of the multifunctional enzyme.

Example 1 - In Vitro Binding of HL60 Cells to Endothelial Cells

Endothelial cells were first passaged onto 96 well plates at a given concentration. The endothelial cells used in the experiment are described in Edgell et al., *Proc. Natl. Acad. Sci. USA* (1983) 80:3734. The cells were incubated at 37°C under a DMEM cell culture medium containing 10% fetal calf serum and under a 5% CO_2 atmosphere. Then, the medium was removed and replaced with 100 μl (microliter) of a suspension of 200,000 HL60 cells (a human lymphocyte cell line, available from the European Cell Culture Bank under ECACC Accession No. 85011431) in RPMI medium containing 10% fetal calf serum. The cells were incubated for 30 minutes. After this, the medium was removed and the adherent cells were washed two times with DMEM medium. The relative adherence of the HL60 cells was measured by measuring the difference in optical density at 450nm between the plates on which the cells were co-incubated and plates having endothelial cells alone.

The effect of TNFα was measured by adding TNFα at 1500 units/ml to the endothelial cells 4 hours before the incubation with HL60 cells. The effect of antibody to E-selectin was measured by adding 25μg/ml of monoclonal antibody BBAZ (R&D Systems Europe, Oxford, England) to the HL60 cells. The results of the experiments were:

Expt. No.	HL60 Cells	Endothelial Cells	Absorbance*
1	no treatment	no treatment	0.324
2	no treatment	pretreated with TNFα	0.444
3	added in the presence of mAb to E-selectin	pretreated with TNFα	0.357

*increase over absorbance of endothelial cells alone

The effects of the krill multifunctional hydrolase on this system were measured by:

1. measuring the effect of adding to the endothelial cells 92.3μg/ml krill multifunctional hydrolase (prepared as in Example 1C of WO 96/24371 (Phairson Medical)) together with the HL60 cells;
2. after pretreating the endothelial cells with TNF for 2 hours, adding 92.3 μg/ml krill multifunctional hydrolase and incubating for 2 more hours prior to the addition of HL60 cells; or
3. pretreating the HL60 cells with 92.3 μg/ml krill multifunctional hydrolase prior to adding the HL60 cells to the plates of endothelial cells.

The results of these experiments were as follows:

Expt. No.	HL60 Cells	Endoth Cells	Absorb-ance*
4	Multifunctional enzyme added simultaneously with cells	pretreated with TNFα	0.425
5	no treatment	Four hours pretreatment: 0-4h TNFα 2-4h multifunctional enzyme	0.247
6	pretreated with multifunctional enzyme for 2h	pretreated with TNFα	.0.160
7	pretreated with multifunctional enzyme for 2h	Four hours pretreatment: 0-4h TNFα 2-4h multifunctional enzyme	0.059

*increase over absorbance of endothelial cells alone.

To confirm these results, the number of adhering HL60 cells were counted by removing them from the plate and counting the cells. The number of HL60 cells was determined by subtracting the cell numbers for control plates having only endothelial cells. These counting results mirrored the optical density results, as follows:

EXPERIMENT	HL60 CELL NUMBER
1	32,590
2	43,990
3	35,730
4	42,190
5	25,280
6	17,010

These adherence studies show that krill hydrolase destroyed the cell-surface ligand and acceptor molecules that facilitate cell-adhesion.

Example 2- Activity Against Certain Cell-Surface Adhesion Molecules

Freshly isolated T-cells from the thymus of a C57BL/6 mouse were washed three times with serum-free medium. 1 ml aliquots of the cells containing 5 - 10 X 10^6 cells were treated at 37°C for 4 hours with 0, 100 or 500 μg/ml of the krill-derived multifunctional hydrolase prepared according to Example 1B dissolved in serum-free medium. Resulting cells were labeled with one of fluorescent antibodies identified below:

Antibody	Source
CD4-PE	Boehringer Mannheim, Laval, Quebec
CD8-Red613	GIBCO, Long Island, New York
ICAM-1	PharMingen, San Diego, CA
ICAM-2	PharMingen, San Diego, CA
CD44	PharMingen, San Diego, CA
H-2K	PharMingen, San Diego, CA

The amount of antibody binding was determined using a fluorescence-activated cell sorter. From the results, it was determined that the order of sensitivity to inactivation or removal by the hydrolase was CD4, CD8 < ICAM-2 (CD102) < CD44 < ICAM-1 (CD54) < H-2K. Using these same methods with appropriate cells, including endothelial cells, including the s-end-1 endothelial cell line (Kinashi et al., *J. Leukocyte Biol.* 57: 168, 1995) and T-cells isolated from the thymuses of C57BL/6 mice, it was determined

that the VCAM-1, CD28, CD 31 and asialo GM1 ceramide markers are sensitive to the hydrolase. The antibodies used to make these determinations were:

Antibody Specificity	Source
VCAM-1	PharMingen, San Diego, CA
CD28	PharMingen, San Diego, CA
CD31	PharMingen, San Diego, CA
asialoGM1	Wako Bioproducts, Richmond, VA

In some cases, binding was detected with a labeled second antibody, for instance, binding of the asialo GM1 antibody was detected with FITC-labeled Fab fragments that were specific for rabbit IgG (heavy and light chains), which was obtained from Caltag Laboratories, San Francisco, CA.

Example 3 - Timecourse of Cell Surface Recovery of Adhesion Molecules

O-11.10 T-cell hybrids (this cell line is described by Shimonkevitz et al., *J. Experimental Med.* 158: 303, 1983) were treated with 500 μg/ml of the krill-derived multifunctional hydrolase prepared as described in Example 1B of WO 96/24371 (Phairson Medical) and tested for the CD4 marker as described in Example 2. Immediately after the treatment, well less than 1% of the amount of CD4 found in the controls was found on the hydrolase-treated cells. 48 hours later, the levels in treated cells were the same as those in untreated cells.

Example 4 - GVHD and Bone Marrow Transplantation

Materials and Methods

Mice

Female C57BL/6 (H-2^b), DBA/2 (H-2^k), and (C57BL/6 x DBA/2)F_1 mice (abbreviated BDF1 mice, H-$2^{b,k}$) were purchased from Charles River Laboratories (St-Constant, Quebec, Canada). Animals were housed microisolator cages at the Institut Armaud Frappier specific-pathogen free facility. At the time of bone marrow transplantation, donors and recipients were 6 to 8 weeks of age in the first series of experiments, and 5 to 10 weeks of age in the second series of experiments.

Bone marrow transplantation (BMT)

Recipients were given a single dose of 700 r total body irradiation 2-4 hours before transplantation from a ^{60}Co irradiator. Irradiated recipients received, as a source of T-cells, a single intravenous injection via the tail vein of 5 x 10^6 bone marrow cells and 5 x 10^6 spleen cells. Spleens, femurs, and tibias were aseptically collected from

euthanized donors and placed in ice-cold Hank's balanced salt solution (HBSS). Spleens were pressed through sterile wire mesh to obtain single cell suspensions which were further treated with Tris-buffered ammonium chloride or sterile distilled water (hypotonic lysis) to eliminate erythrocytes. Bone marrow cells were flushed with a needle and a syringe from femoral and tibial cavities of donor mice and collected. All cell suspensions were washed twice with HBSS before use.

Treatment of spleen cells with proteases

In some experiments, spleen cells were treated with proteases prior to injection in irradiated recipients. The purified krill-derived multifunctional enzyme ("PHM protease") was obtained from Phairson Medical Ltd (Batch No. PS-3; London, England) in a freeze-dried form and reconstituted with sterile serum-free RPMI medium. Cod trypsin was obtained in a liquid form from Dr. Jon Bjarnason (University of Iceland) (Lot NO. 27.11.95) and had a specific activity of 173 U/mg (CBZ-GRPpNA hydrolyzing activity). The enzyme was dialyzed against 1 L of serum-free RPMI stored in frozen aliquots. Papain was obtained from Sigma (St. Louis, MO). In the first series of experiments, donor C57BL/6 spleen cells were treated with 50 μg/ml of PHM in serum-free RPMI medium for 2 h at 37°C, whereas DBA/2 cells were treated with 20 μg/ml of PHM in serum-free RPMI medium for 2 h at 37°C. In the second series of experiments, donor C57BL/6 spleen cells were treated with 20 μg/ml of protease (e.g. cod trypsin, papain, or PHM) in serum-free RPMI medium for 1 h at 37°C. Controls included spleen cells incubated without proteases in serum-free RPMI for the same period of time at 37°C. Cells were then washed twice in serum-free medium and counted using Trypan blue staining,

Flow cytometry

Spleen cells from C57BL/6 mice were stained with saturating amount of PE-labeled anti-CD4 (Pharmingen, San Diego, CA) and Red-613-labeled ant-CD8 antibodies (GIBCO-BRL, Mississaugua, Ont, Canada) obtained commercially. The stained cells were analyzed on a Coulter XL-MCL laser flow cytometer (Hialeah, FL).

Experimental design

The experiments were designed to investigate the impact of protease treatment of splenocytes on the prevention of lethal GVHD. Donor cells from C57BL/6 (H-2^h), or DBA/2 (H-2^k), were injected in semi-allogeneic BDFl (H-$2^{h,k}$) recipients. In this model,

rejection of the bone marrow graft is not possible as H-2^b or H-2^k cells are recognized as self by the BDF1 recipients. GVHD is induced either upon allorecognition of H-2^k antigens expressed by the antigen presenting cells of the recipients following injection of C57BL/6 (H-2^b) donor T-cells, or upon allorecognition of H-2^b antigens on the antigen presenting cells of the recipients following injection of DBA/2 (H-2^k) donor T-cells. In this model of GVHD, mature donor T-cells are mixed with the bone marrow inoculum since the number of donor T-cells in the marrow inoculum is insufficient to induce reproducible and acute GVHD (Ushiyama et al. 1995). Since all nucleated cells express H-2 antigens, the attack of the donor T-cells can be severe, and kill the animals (acute GVHD). Sometimes depending on the H-2 mismatch between donor and recipients, the GVH reaction is mild, and does not kill the recipients (e.g. chronic GvHD). Mice were observed periodically for clinical signs of the disease, and their weight measured twice a week.

<u>*Results*</u>

The first-series of experiments:

PHM can cleave several cell surface adhesion molecules from the surface of T lymphocyte cell lines in vitro, including CD4, CD8, CD62L, CD54, and others. Thus, PHM can cleave receptors from the surface of freshly isolated splenocytes of C57BL/6. The cleavage of the CD4 and CD8 molecules by PHM was dose-dependent. When PHM is used at concentrations above 20 µg/ml, we found that PHM completely removed the expression of CD4 or CD8 from the surface of splenocytes. Splenocytes from C57BL/6 mice were incubated with the indicated concentrations of PHM for 1 h at 37°C in serum-free RPMI medium. Cells were then stained with specific antibodies to CD4 or CD8, and analyzed by laser flow cytometry.

Since PHM can remove any expression of CD4 or CD8 at the surface of splenocytes, and since both CD4 and CD8 have been reported to play a key role in the induction of GVHD, the question of whether *ex vivo* treatment of lymphoid cells with PHM could reduce the adverse effect of GVHD in a murine model of severe GVHD was investigated. In the first series of experiments, a group (n = 6) of lethally irradiated BDF1 (C57BL/6 x DBA/2, H-$2^{b,k}$) recipients were reconstituted with 5 x10^6 bone marrow and 5 x 10^7 splenocytes from C57BL/6 (H-2^b) mice. In this model, spleen cells were added to the bone marrow inoculum since there is often not enough T-cells in the

bone marrow to induce severe GVHD. Under these conditions, BDF1 recipients reconstituted with C57BL/6 bone marrow cells and splenocytes did not survive allogeneic BMT, as most of recipients died within 4 weeks post-transfer (untreated group, Figure 1A). In contrast, C57BL/6 inoculums of bone marrow cells + splenocytes from did not induce a GVH reaction when inoculated into histocompatible, lethally irradiated C57BL/6 recipients (syngeneic control, Figure 1A). *Ex vivo* treatment of C57BL/6 splenocytes with PHM (50 μg/ml for 2 h at 37°C) was sufficient to prevent, at least partially, the ability of splenocytes to induce lethal GVHD in BDFl recipients. Whereas BDF1 recipients receiving normal C57BL/6 splenocytes died within 4 weeks post-transfer, most of the recipients (4/6) receiving PHM-treated splenocytes mixed with BMC survived up to 60 days post-transfer. In Figure 1, controls included recipients receiving untreated semi-allogeneic splenocytes (untreated) and recipients receiving syngeneic PHM-treated (50 μg/ml for 2 h at 37°C) splenocytes (Syngeneic + PHM.). Figure 1B illustrates mean survival times (MST) of the three different groups of recipients.

GVHD is associated with severe weight loss. BDFl recipients receiving C57BL/6 bone marrow mixed with splenocytes suffered irreversible and severe weight loss while recipients receiving histocompatible bone marrow inoculum occasionally lost some weight shortly after the transfer due to the irradiation, but subsequently showed signs of recovery as indicated by a continuous gain of weight. BDFI recipients reconstituted with C57BL/6 bone marrow cells and the PHM-treated splenocytes also recovered from the initial weight loss associated with the irradiation then underwent a period of gradual weight loss between day 15 and they 30. After day 30, however, these BDFl recipients started to fully recover and most of these recipients survived and gained weight. For these results, weights of individual mice were monitored twice a week for each BDF1 recipient receiving C57BL/6 semi-allogeneic bone marrow cells mixed with (A) untreated splenocytes, (B) PHM-treated splenocytes, or (C) syngeneic, PHM-treated splenocytes.

In murine models of GVHD, it is sometimes difficult to accurately predict the onset of GVHD, irrespective of the level of histocompatibility between donors and recipients. In the above experiments, the combination of C57BL/6 with BDF1 recipients was indeed a good model of GVHD. To obtain a second model of GVH, we also

reconstituted the BDF1 recipients with DBA/2 bone marrow cells mixed with DBA/2 splenocytes with a similar inoculum of bone marrow cells and splenocytes. However, reconstitution of DBF1 recipients with DBA/2 BMC did not lead to an acute and lethal GVHD. Only one BDF1 recipients died after bone marrow graft. Again, in this model, a temporary weight loss was observed shortly (< 10 days), but all recipients survived and showed continuous gain of weight thereafter.

The second series of experiments:

Since CD4 and CD8 play a crucial role as accessory signals in the T-cell response to allogeneic antigens, treatment of splenocytes with PHM could prevent lethal GVHD by removing all cell surface expression of both receptors at the surface of the treated splenocytes prior to the transfer. To gain further insight into this possibility, a second series of experiments were conducted using three proteases: cod trypsin, PHM and papain. Whereas both cod trypsin and PHM can efficiently cleave CD4 and CD8, papain cannot cleave either receptor efficiently.

Lethally irradiated BDF1 recipients were therefore reconstituted as in the first series of experiments, i.e. using 5 X10^6 bone marrow and 5 X 10^7 splenocytes. Additional experiments with the spleen of C57BL/6 donor mice showed that efficient cleavage of CD4 and CD8 at their surface could be obtained using a milder treatment of splenocytes with proteases. Thus, the time of incubation with proteases was lowered from 2 h to 1 h, keeping the temperature of incubation at 37°C, and the dose of proteases for *ex vivo* treatment was lowered to 20 µg/ml. Again treatment of splenocytes with a protease significantly reduced the mortality associated with histoincompatible engraftment (Figure 2). At four weeks post-transfer, most of the recipients having received an inoculum of splenocytes treated with a protease had a significantly higher percentage of survival as compared to those reconstituted with untreated splenocytes. The most significant effect was observed with treatment using cod trypsin, as most of the BDF1 mice reconstituted with cod trypsin-treated splenocytes survived the histoincompatible bone marrow graft from C57BL/6 donors. At day 43 post transfer, most of the these BDFl recipients had stabilized their weight, while some show significant gain of weight.

In the current experiments, lethal GVHD has been prevented. Engraftment has been demonstrated, confirming the indication of successful engraftment that follows

from the observation that irradiated recipients that do not receive a bone marrow graft die within one week post-transplantation.

Example 5 - T-Cell Proliferation in Response to Mitogen

Peripheral blood mononuclear cells (PBMCs) were isolated from human blood. 1 X 10^5 PBMCs per well were incubated for 72 h at 37°C in medium supplemented with 10% human serum under 5% CO_2, in the presence of various dilutions of phyto-haemagglutinin (PHA) in the presence (or absence) of a dilution of cod trypsin (diluted 1:100 or more from a 173 U/ml stock solution). At 54 h, the wells were pulsed with ^{3}H-thymidine. After cell harvest, ^{3}H uptake was measured, with each experimental point determined from triplicate cultures. The result was that a dose-responsive diminishment in ^{3}H uptake was seen, with the diminishment first apparent at the 1:1000 dilution and with the 1:100 dilution showing negligible uptake.

A parallel experiment with PHM at best a small effect, but later analysis showed that the starting dilution corresponded to about the 1:10,000 dilution of cod trypsin, such that no effect would have been expected.

Example 6 - T-Cell Proliferation to Alloantigen (Mixed Lymphocyte Reaction)

1 X 10^4 PBMCs (responders) per well were incubated with 1 X 10^5 irradiated allogeneic PBMCs (stimulators). The responder PBMCs and stimulator PBMCs were isolated from the blood of different humans. Cells were incubated under the culture conditions of Example 5 for six days in the presence or absence of cod trypsin (various dilutions). Again a ^{3}H-thymidine pulse was used to generate an uptake indicator of mitogenic activity. The results, from triplicate wells, showed a dose response, with the first significant reduction seen at a 1:10,000 dilution, with the 1:100 dilution showing negligible ^{3}H uptake.

Example 7 - Skin Transplantation in Murine Model

First Protocol

Preparation of Balb/c cells: Spleen cells are harvested, red blood cells removed, and T-cell depleted by magnetic separation with anti-Thy-1 (CD90) antibodies (an antigen specifically expressed on all T cells). T-cell-depleted Balb/c stimulator cells (containing mostly B cells and macrophages, and some dendritic cells, and few NK cells) are resuspended in serum-free RPMI (Russell Park Memorial Institute) at 2 x10^6 cells /ml. PHM or cod trypsin (1, 5, 50 µg/ml final concentration) is added and cells

incubated for 2 hours at 37°C . The reaction with the protease is stopped by adding fetal calf serum (FCS - 10% final concentration). Cells are added to a F-25 flask (10^6 cells/0.5 ml/6.1 cm, i.e. equivalent to 106/24-well plate). An aliquot of cells is used to confirm cleavage by flow cytometric analysis with anti-B7 and anti-CD40 antibodies. Controls include stimulator cells incubated in serum-free medium without PHM.

Preparation of C3H cells: Spleen cells (N.B. 15% of spleen cells are T cells) are harvested as a source of T-cells, red blood cells are removed. Cells are counted and added to F-25 flasks containing PHM-treated C3H stimulator cells at given ratios of 1:1. Cells are incubated for 24 hours at 37°C.

Separation of *ex vivo* tolerized T cells from co-culture: all T-cells from co-culture are isolated using antibodies to Thy-1 (CD90) by magnetic separation (since both CD4 and CD8 are responsible for tolerization (Blazar et al., 1996)). This procedure allows isolation of C3H-T cells away from Balb/c stimulator cells. T-cells are washed twice with PBS to remove any trace of PHM, and the cell concentration adjusted to 108/ml.

Induction of tolerance: Recipient C3H mice are injected iv with 1, 10, and 100 x 10^6 donor cells. Challenge with tail skin from Balb/c mice are done 24, 48 or 72 hours later. Skin transplant, as is known in the art, includes cutting the tails of donor animals, cleaning and trimming the skin (3-4 grafts within a tail), preparing the recipient (anaesthesia), trimming a graft bed, and suturing. Inhibition of rejection is measured 10-12 days post-transplant.

Second Protocol

Preparation of C3H cells: Spleen cells are harvested, red blood cells removed, and T-cell depleted by magnetic separation with anti-Thy-1 (CD90) antibodies (an antigen specifically expressed on all T cells). T-cell-depleted stimulator cells (containing mostly B cells and macrophages, and some dendritic cells, and few NK cells) are resuspended in serum-free RPMI (Russell Park Memorial Institute) at 2 x10^6 cells /ml. PHM or cod trypsin (1, 5, 50 μg/ml final concentration) is added and cells incubated for 2 hours at 37°C. The reaction with the protease is stopped by adding fetal calf serum (FCS - 10% final concentration). Cells are added to a F-25 flask (10^6 cells/0.5 ml/6.1 cm, i.e. equivalent to 106/24-well plate). An aliquot of cells is used to confirm cleavage by flow cytometric analysis with anti-B7 and anti-CD40 antibodies. Controls include stimulator cells incubated in serum-free medium without PHM.

Preparation of Balb/c cells: Spleen cells (N.B. 15% of spleen cells are T cells) are harvested as a source of T-cells, red blood cells are removed. Cells are counted and added to F-25 flasks containing PHM-treated C3H stimulator cells at given ratios of 1:1. Cells are incubated for 24 hours at 37°C.

Separation of *ex vivo* tolerized T cells from co-culture: all T-cells from co-culture are isolated using antibodies to Thy-1 (CD90) by magnetic separation (since both CD4 and CD8 are responsible for tolerization (Blazar et al., 1996)). This procedure allows isolation of Balb/c T-cells away from C3H stimulator cells. T-cells are washed twice with PBS to remove any trace of PHM, and the cell concentration adjusted to 108/ml.

Induction of tolerance: Recipient C3H mice are injected iv with 1, 10, and 100 x 10^6 T-cells. Challenge with tail skin from Balb/c mice are done 24, 48 or 72 hours later. Skin transplant, as is known in the art, includes cutting the tails of donor animals, cleaning and trimming the skin (3-4 grafts within a tail), preparing the recipient (anaesthesia), trimming a graft bed, and suturing. Inhibition of rejection is measured 10-12 days post-transplant.

Example 8 - Effect of Serum

1 X 10^5 PBMCs per well were incubated for 72 h at 37°C in in medium supplemented with 10% human serum under 5% CO_2, in the presence of an appropriate dilution of PHA (selected based on Example 5) in the presence (or absence) of cod trypsin (diluted 1:100) and 0%, 1%, 2%, 5% or 10% human AB serum. The wells were pulsed with ^{3}H-thymidine. After cell harvest, ^{3}H uptake was measured, with each experimental point determined from triplicate cultures. The result was that 1% or 2% serum nearly doubled the hydrolase-induced inhibition of the mitogenic response, while 5% or 10% serum nearly halved the hydrolase-induced inhibition of the mitogenic response.

Example 9 - Removal of Cell-Surface Proteins

Human PBMCs per incubation were incubated at 37°C for 5 h in the presence or absence of 6 μg/ml PHM or 200 μg/ml cod trypsin. The effects of these treatments on various surface markers were measured by flow cytometry with fluorescently labeled antibodies and summarized by percent of cells having the marker and median fluorescence. **Figure 3A** shows the results for CD3, CD4 and CD8, with open squares

representing the results with no enzyme, open triangles for the cod trypsin treatment, and open circles for the PHM treatment. The results for CD25 and CD28 are in **Figure 3B**; for CD11a, CD49a and CD54 in **Figure 3C**.

Example 10 - Protease Comparisons

Aliquots of T-lymphocytes (2 X 10^6) were incubated in 0.5 ml of RPMI 1640 culture medium at 37°C for 2 h in the presence of 2 mcg/ml or 20 mcg/ml of a protease. After incubation, the protease was removed by washing the cells using low speed centrifugation. The effects on surface molecules was quantitated with fluorescent-labeled monoclonal antibodies and fluorescence measured on individual cells by flow cytometry. The quantity is derived from 10,000 cells per measurement. The 2 mcg/ml results are shown in **Figure 4** for CD62L (panel A), CD8 (panel B), CD54 (panel C), CD11a (panel D), CD102 (panel E), CD4 (panel F) and CD31 (panel G). The proteases were:

Enzyme	Source
PHIM	see, U.S. Patents 5,945,102 and 5,958,406
Kamchatka protease	Anawa, Wangen, Switzerland
Cod chymotrypsin	Atlantic cod, see, Aseirsson and Bjarnason, *Comp. Biochem. Physiol.* 99B:327-335, 1991
Cod trypsin	Atlantic cod, see, *European J. Biochem.* 180: 85-94, 1989
Cod collagenase 3	Atlantic cod, see, Aseirsson and Bjarnason, *Comp. Biochem. Physiol.* 99B:327-335, 1991
Cod cryotin IV	Atlantic cod, see, Professor Bjarnason, Univ. or Ra
Cod elastase	Atlantic cod, see, Aseirsson and Bjarnason, *Biochem. Biophys. Acta.* 1164:91-100, 1993
Papain	Sigma Chemical, St. Louis
Bromelain	Sigma Chemical, St. Louis
Subtilisin	Sigma Chemical, St. Louis
Tunisin	Gaiker, Zamudio, Spain
Collagenase F	Sigma Chemical, St. Louis

The 20 mcg/ml results are shown in panels A-G of **Figure 5**.

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The protein sequences described herein and in documents identified herein have been carefully sequenced. However, those of ordinary skill will recognize that nucleic acid sequencing technology can be susceptible to inadvertent error. Those of ordinary skill in the relevant arts are capable of validating or correcting these sequences based on the ample description herein of methods of isolating the nucleic acid sequences in

question, and such modifications that are made readily available by the present disclosure are encompassed by the present invention. Furthermore, those sequences reported herein are believed to define functional biological macromolecules within the invention whether or not later clarifying studies identify sequencing errors. Moreover, please note that sequences recited in the Sequence Listing below as "DNA" or under some other apparently restrictive nomenclature, represent an exemplification of the invention, since those of ordinary skill will recognize that such sequences can be usefully employed in polynucleotides in general, including ribopolynucleotides.

All publications and references, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference in their entirety as if each individual publication or reference were specifically and individually indicated to be incorporated by reference herein as being fully set forth. Any patent application to which this application claims priority is also incorporated by reference herein in its entirety in the manner described above for publications and references.

While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations in the preferred devices and methods may be used and that it is intended that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the claims that follow.

What is claimed:

1. A method of preventing or ameliorating transplantation rejection reactions, where the transplantation can be of immune cells or another tissue, the method comprising:

treating a source of immune cells with a rejection preventing or ameliorating effective amount of a hydrolase or mixture of hydrolases; and

administering the treated immune cells to a recipient animal.

2. The method of preventing or ameliorating transplantation rejection reactions of claim 1, further comprising:

contacting the treated immune cells, which cells are obtained from a recipient animal, with second cells of a donor animal; and

transplanting a tissue from the donor animal to the recipient animal.

3. The method of preventing or ameliorating transplantation rejection reactions of claim 1, further comprising:

administering the immune cells with a cell surface adhesion molecule binding effective amount of an antibody that binds one of CD4, CD8, CD25 (IL-2 receptor alpha chain), CD28, CD152 (CTLA-4), an integrin, CD154, CD40 and CD80.

4. The method of preventing or ameliorating transplantation rejection reactions of claim 1 comprising:

isolating from a source of immune cells taken from a donor (a) a fraction enriched in mature T-cells and (b) a fraction containing immune cell precursor cells;

treating the mature T-cells of fraction (a) with a rejection preventing or ameliorating effective amount of a hydrolase or mixture of hydrolases; and

administering the mature T-cells of fraction (a) and the cells of fraction (b) to a recipient.

5. The method of claim 4, wherein the hydrolase treated mature T-cells are contacted with cells of fraction (b) prior to administration to the recipient.

6. A method of preventing or ameliorating allergic, autoimmune or transplantation rejection reactions comprising:

treating a source of immune cells taken from a recipient with an allergic, autoimmune or transplantation rejection preventing or ameliorating effective amount of a hydrolase or mixture of hydrolases;

contacting the treated source of immune cells with cells from the donor animal or with a substance that induces the allergic reaction or which contains autoimmune epitopes;

transplanting the donor organ into the recipient; and

administering the treated cells into the recipient.

7. The method of claim 6, wherein the treated cells include mature T-cells.

8. The method of claim 6, further comprising

administering the treated immune cells with a cell surface adhesion molecule binding effective amount of an antibody that binds one of CD4, CD8, CD25 (IL-2 receptor alpha chain), CD28, CD152 (CTLA-4), an integrin, CD154, CD40 and CD80.

9. A method of preventing or ameliorating allergic, autoimmune or transplantation rejection reactions with a hydrolase, comprising:

identifying the hydrolase or mixture of hydrolases as a hydrolase or mixture of hydrolases (a) effective to induce tolerance in immune cells to a substance against which the immune cells were previously reactive, or (b) with a relative selective preference for disabling signal 2 and/or signal 1;

treating immune cells or immune cell precursors with the hydrolase or mixture of hydrolases; and

administering the treated cells to a mammal.

10. The method of claim 9, wherein the hydrolase has a relative selective preference for removing, destroying, inactivating or disabling at least one of at least one of CD4, CD8, CD25, CD28, ICAM-1 (CD54), CD152, an integrin, CD154, CD40 and CD80 over removing, destroying, inactivating or disabling TcR.

11. The method of claim 9, wherein the hydrolase has a relative selective preference for removing, destroying, inactivating or disabling at least one of at least one CD4, CD8, CD28, ICAM-1 (CD54), CD11a, CD49d and CD154 over removing, destroying, inactivating or disabling TcR.

12. The method of claim 9, wherein the hydrolase has a relative selective preference for removing, destroying, inactivating or disabling at least two of CD4, CD8, CD28 and CD154 over removing, destroying, inactivating or disabling TcR.

13. A method of identifying a hydrolase for use in preventing or ameliorating allergic, autoimmune or transplantation rejection reactions, comprising:

identifying a relative selective preference of one or more hydrolases for removing, destroying, inactivating or disabling at least one of CD4, CD8, CD25, CD28, ICAM-1 (CD54), CD152, an integrin, CD154, CD40 and CD80 in contrast to removing, destroying, inactivating or disabling TcR; and

selecting a hydrolase or mixture of hydrolases with a relative selective preference for removing, destroying, inactivating or disabling at least one of CD4, CD8, CD25, CD28, ICAM-1 (CD54), CD152, an integrin, CD154, CD40 and CD80 in contrast to removing, destroying, inactivating or disabling TcR.

14. A method of preventing or ameliorating transplantation rejection reactions comprising treating the donor tissue with a rejection reaction preventing or ameliorating effective amount of a hydrolase or mixture of hydrolases, wherein the hydrolase or mixture of hydrolases employed is more effective on a molar basis in preventing or ameliorating donor tissue rejection than is the krill multifunctional enzyme.

15. The method of claim 14, further comprising treating the donor tissue *ex vivo*.

16. The method of claim 14, wherein the hydrolase or mixture of hydrolases employed is more effective in removing, destroying, inactivating or disabling one or more of CD4, CD8, CD25, CD28, ICAM-1 (CD54), CD152, an integrin, CD154, CD40 and CD80 than is the krill multifunctional enzyme.

17. The method of claim 14, wherein the hydrolase or mixture of hydrolases employed is more effective in removing, destroying, inactivating or disabling one or more of CD4, CD8, CD28, ICAM-1 (CD54), an integrin, CD154, than is the krill multifunctional enzyme.

18. A method of preventing or ameliorating transplantation rejection reactions comprising treating a donor source of immune cells with a rejection preventing or ameliorating effective amount of a hydrolase or mixture of hydrolases, wherein the hydrolase or mixture of hydrolases employed is more effective on a molar basis in preventing or ameliorating donor tissue rejection than is the krill multifunctional enzyme.

19. The method of claim 18, wherein the hydrolase or mixture of hydrolases employed is more effective in removing, destroying, inactivating or disabling one or more of CD4, CD8, CD25, CD28, ICAM-1 (CD54), CD152, an integrin and GP39 (CD154) than is the krill multifunctional enzyme.

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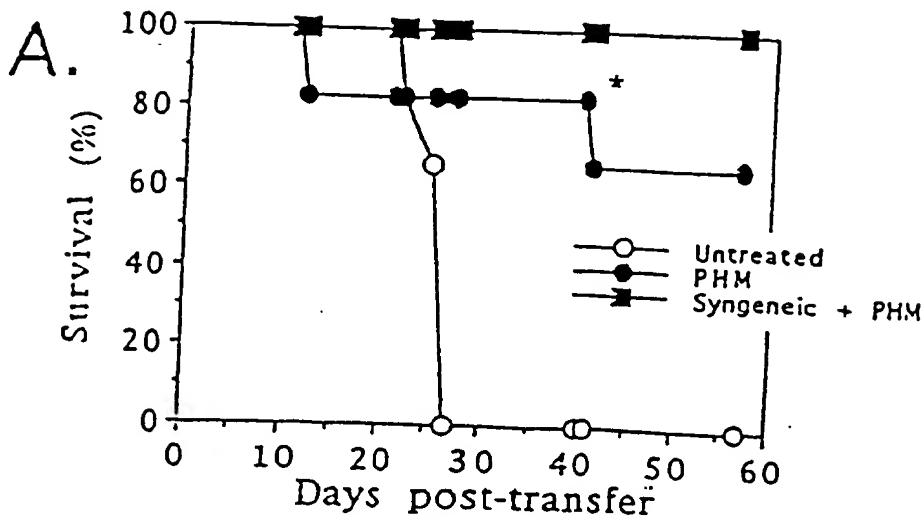

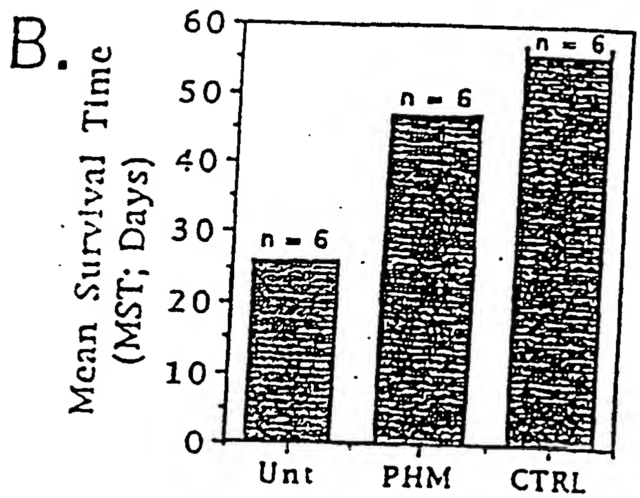

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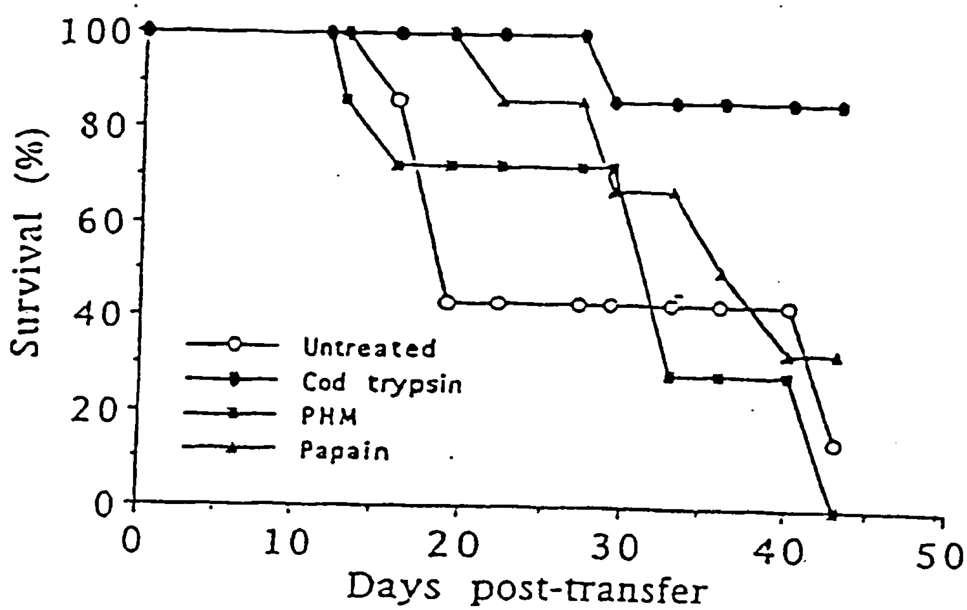
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Untreated
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PHM
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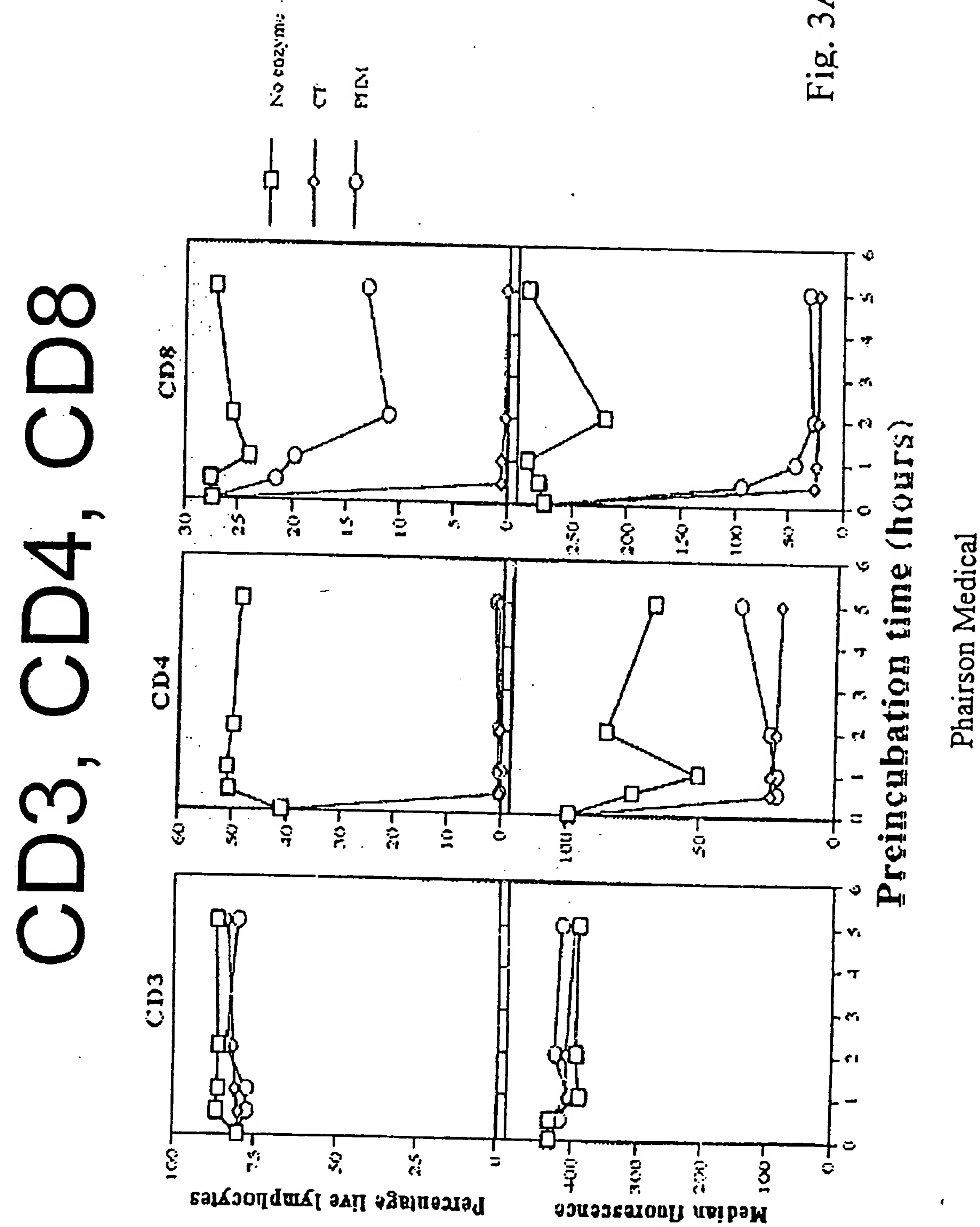
10
20
30
40
50
Days post-transfer

Figure 2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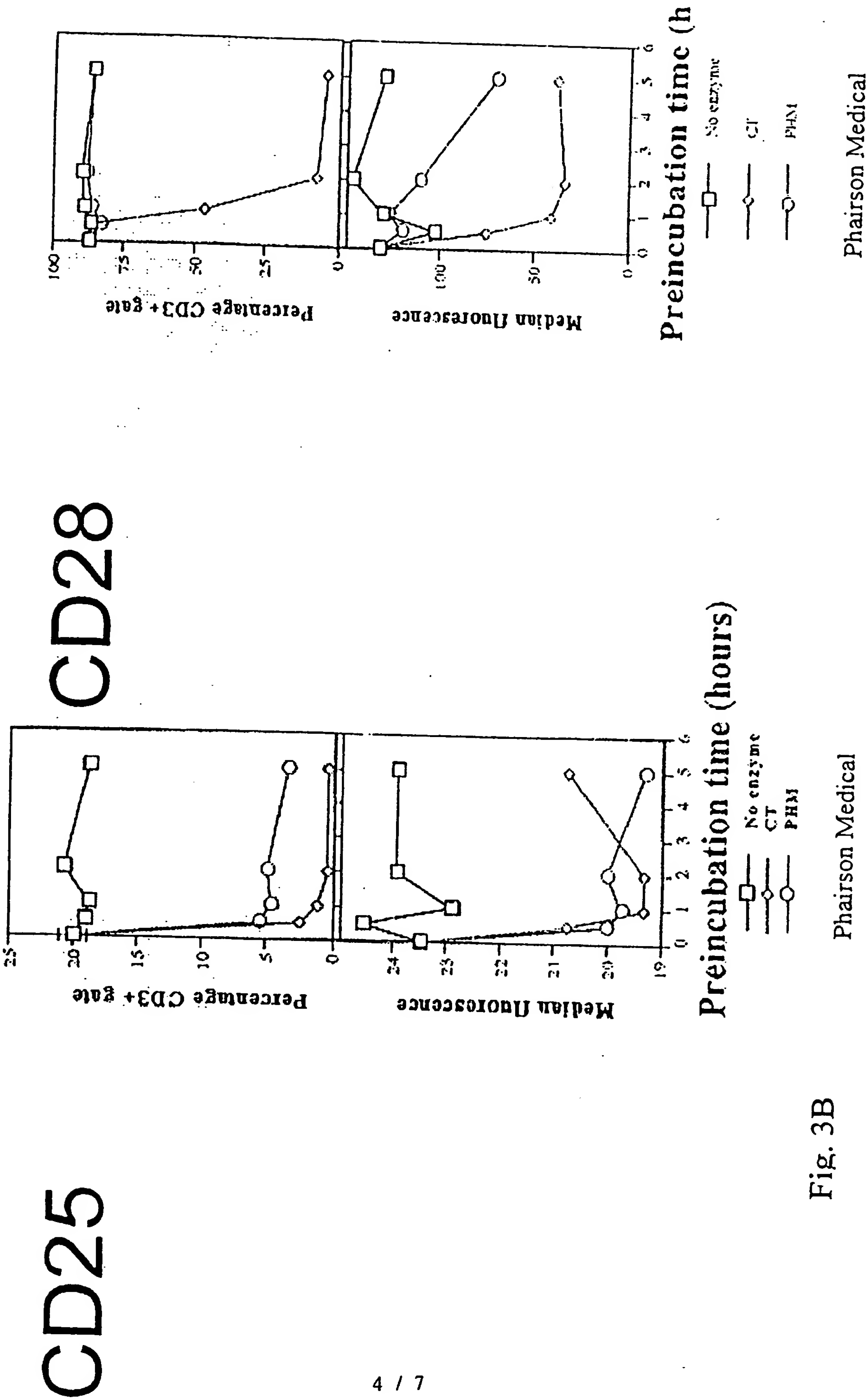
Median fluorescence
Preincubation time (hours)
Phairson Medical

Fig. 3A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Phairson Medical

Fig. 3B

CD11a, CD49d, CD54

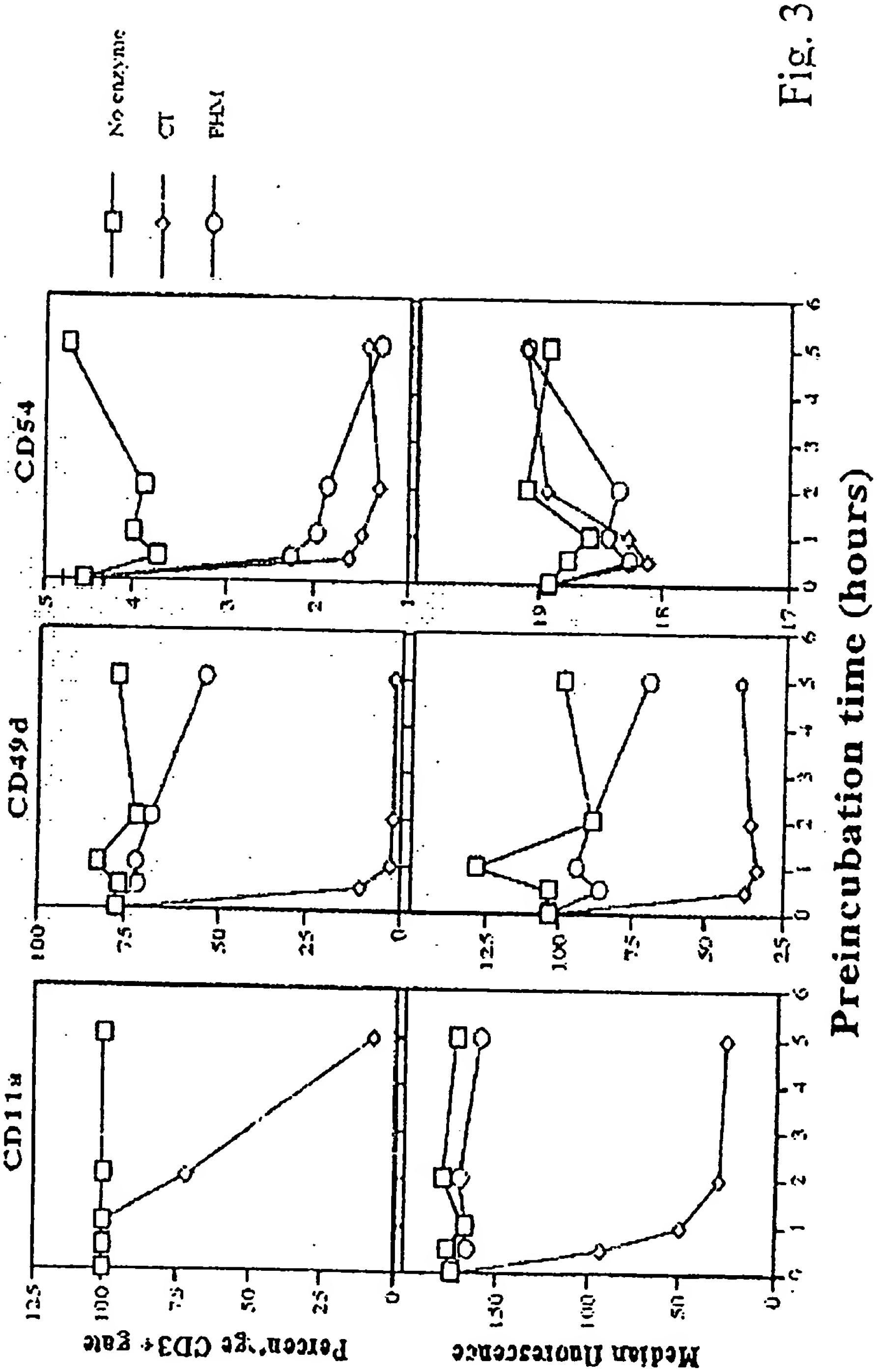

Fig. 3C

Phairson Medical

Figure 1: Cleavage of murine cell surface receptors at a dose of 2 μg/ml.

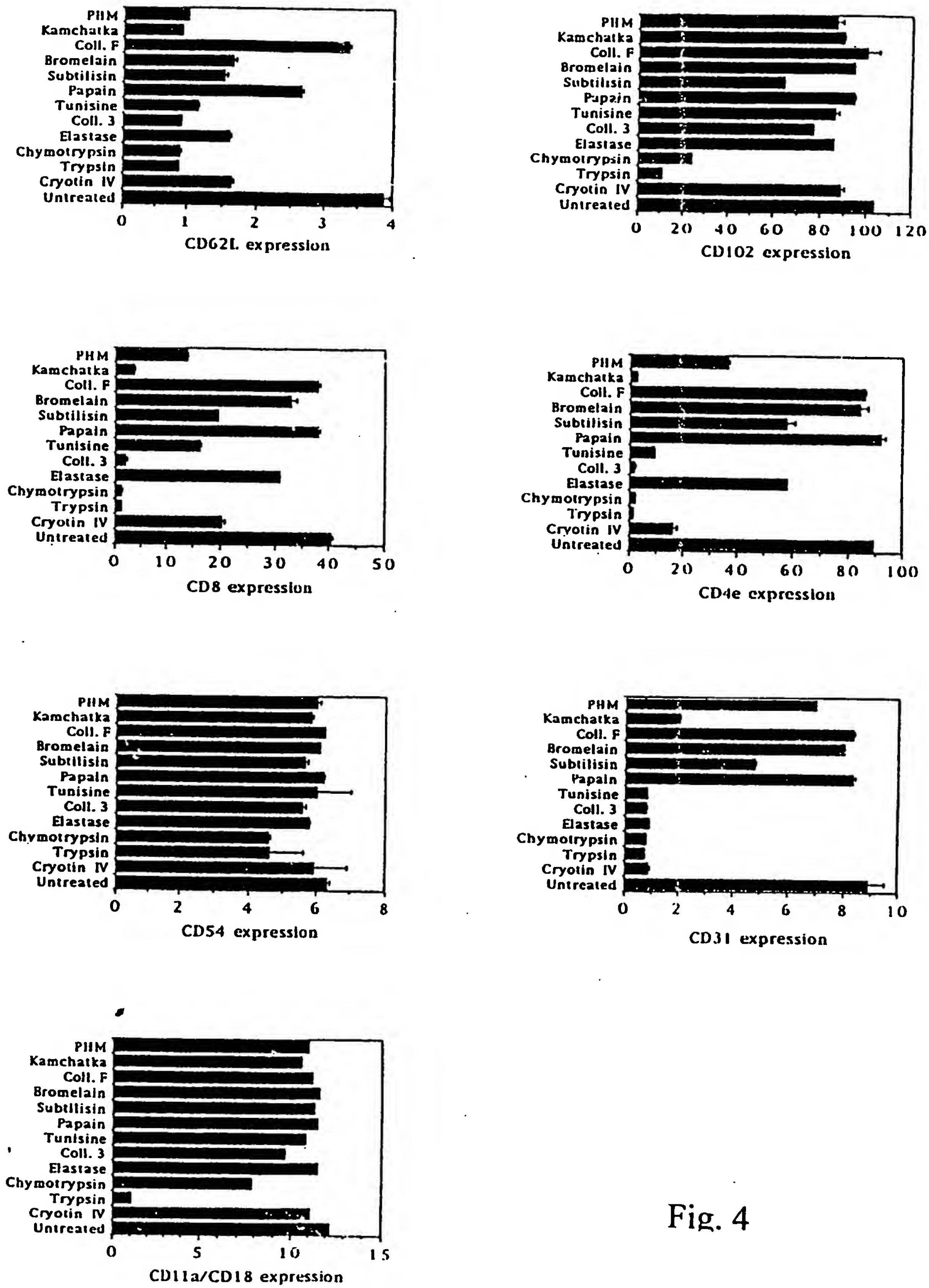

Fig. 4

Figure2: Cleavage of murine cell surface receptors at a dose of 10 μg/ml.

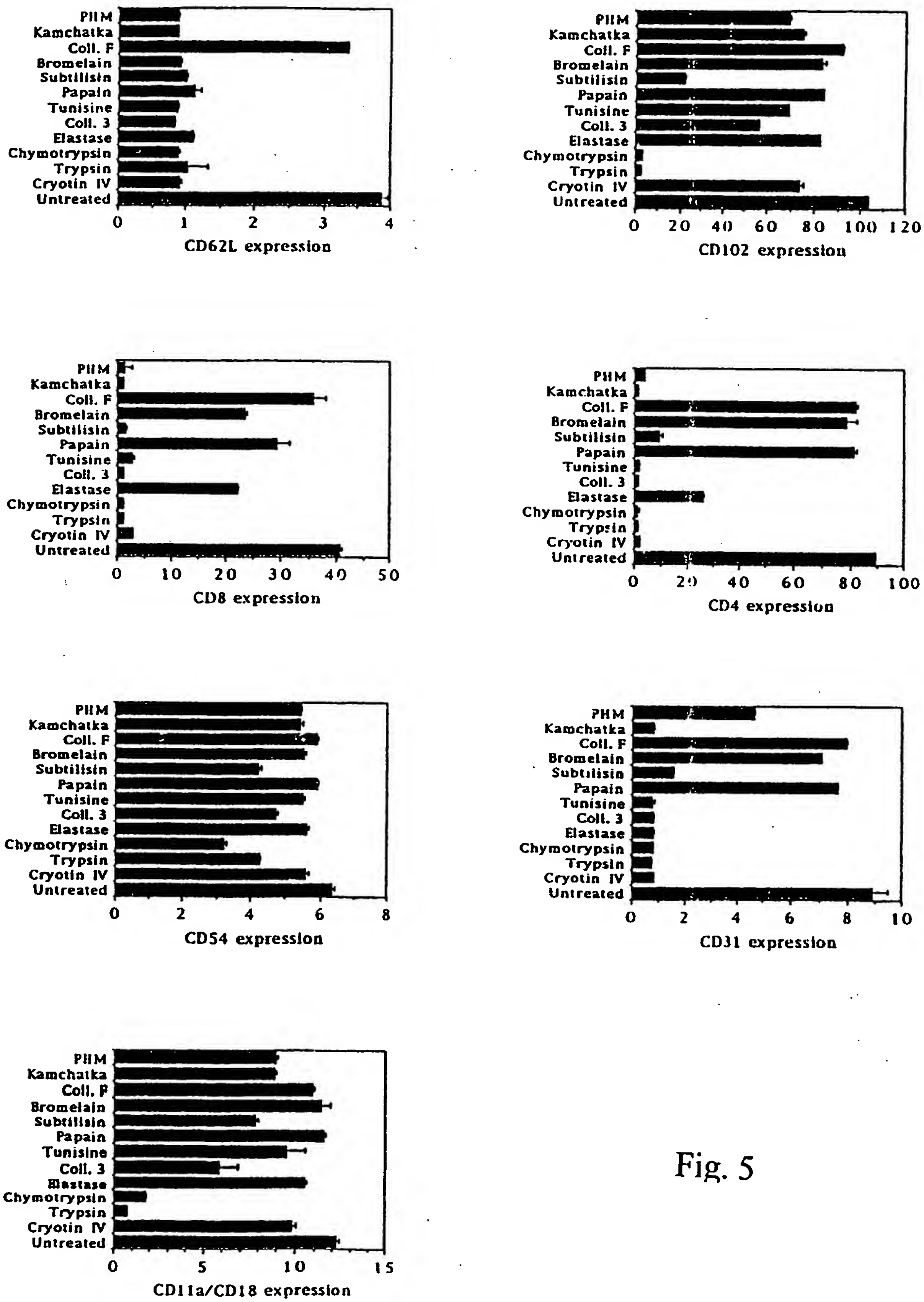

Fig. 5

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Yves St. Pierre

<120> Treatment and Prevention of Immune
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/30818

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 38/43, 38/46, 39/395
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.1, 94.6, 130.1., 133.1, 141.1, 143.1, 144.1, 153.1, 154.1, 173.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, BIOSIS, CA, EMBASE, MEDLINE, USPAT
search terms: hydrolase, krill, phm protease, trypsin, papain, cd4, cd8, cd25, cd28, ctla-4, cd40, cd40 ligand, cd80, b7

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,747,037 A (NOELLE ET AL.) 05 May 1998, see entire document.	1-19
Y	US 5,747,034 A (DE BOER ET AL.) 05 May 1998, see entire document.	1-19
Y	US 5,756,096 A (NEWMAN ET AL.) 26 May 1998, see entire document.	1-19
Y	WO 98/38291 A1 (CORTECS LIMITED) 03 September 1998, see entire document.	1-19

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 MARCH 2000	26 APR 2000

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	PHILLIP GAMBEL
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/30818

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KRADIN et al. Antigen-independent Binding of T-Cells by Dendritic Cells and Alveolar Macrophages in the Rat. AM. REV. RESPIR. DIS. 1989, Volume 139, pages 207-211, see entire document.	1-19
Y	MANNHALTER et al. Modulation of Antigen-Induced T Cell Proliferation by α_2M-Trypsin Complexes. J. Immunol. 15 April 1996, Volume 136, No. 8, pages 2792-2799, see entire document.	1-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/30818

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/94.1, 94.6, 130.1., 133.1, 141.1, 143.1, 144.1, 153.1, 154.1, 173.1

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(54) Title: TREATMENT AND PREVENTION OF IMMUNE REJECTION REACTIONS

(57) Abstract: Provided, among other things, is a method of preventing or ameliorating transplantation rejection reactions comprising treating the donor tissue with a rejection reaction preventing or ameliorating effective amount of a hydrolase that is effective to reduce the amount of one or more cell surface adhesion molecules.

TREATMENT AND PREVENTION OF IMMUNE REJECTION REACTIONS

The present application claims the priority of Provisional Patent Application No. 60/114,147, filed 24 December 1998.

The present invention relates to a treatments to prevent immune rejection reactions, such as graft vs. host disease (GVHD), with a hydrolase effective to remove cell surface adhesion molecules involved in triggering such immune reactions. One embodiment uses a krill-derived multifunctional enzyme and a family of crustacean and fish derived enzymes having substantial structural or functional similarity to the multifunctional enzyme derived from antarctic krill. Another particularly preferred enzyme is an Atlantic cod (*Gadus morhua*) trypsin, particularly that described *European J. Biochem.* 180: 85-94, 1989 and Protein Resource/GenBank Accession No. S03570.

The aquatic or other enzymes that are substantially or functional structurally similar to the krill-derived multifunctional enzyme have the same utility as the krill enzyme. In particular, these enzymes are useful for treating viral infections and other disorders, as outlined for example in U.S. Patent Application Nos. 08/486,820, 08/338,501 (filed November 22, 1994) and U.S. Patents 5,945,102 and 5,958,406.

A WO 96/00082 application of Cortecs Limited describes treating T-cells with bromelain to affect intracellular phosphorylation reactions. The mechanism is said to probably be blockage of "tyrosine phosphorylation of proteins including MAP kinase." The claims of the WO 96/00082 application recite preventing or treating tissue rejection, but no example or protocol for doing so is described. Using antibodies to CD3e and CD28, the authors concluded that "the removal of cell surface molecules by bromelain treatment was not responsible for the reduced cytokine mRNA observed," though *increases* in CD3e and CD28 binding observed "may have contributed" to a proliferative response observed.

A Döring et al., *J. Immunol.* 154: 4842-4850, 1995 article ("Döring") describes the effects of two enzymes found in sputum from cystic fibrosis patients on CD4 and CD8. The enzymes are polymorphonuclear leukocyte-derived proteinase elastase and cathepsin G. Exposure to the polymorphonuclear leukocyte-derived proteinase elastase apparently reduced the cytotoxic response of a T-cell clone. The authors speculate that the effect helps limit tissue damage from the sustained inflammation found in the lungs

of cystic fibrosis patients. Döring does not disclose any treatment of tissue rejection reactions.

Gaciong et al., *Transplantation Proceedings* 28: 3439-3440, 1996 assert that systemic administration of a mixture of bromelain, pancreatic trypsin and a glucoside reduces immune-mediated arteriosclerosis in rat model, where the rats received transplants of allogeneic abdominal aortas. Gaciong et al. describe no tissue-targeted method of preventing or ameliorating transplantation rejection.

SUMMARY OF THE INVENTION

The invention provides a method of preventing or ameliorating transplantation rejection reactions comprising treating the donor tissue with a rejection reaction preventing or ameliorating effective amount of a hydrolase (which can be a mixture of hydrolases), such as a protease. Without limiting the invention to theory, one indication that a hydrolase is appropriate for use in the invention is that such hydrolase is effective to reduce (e.g., remove, destroy, inactivate or disable) the amount of one or more cell surface adhesion molecules. For example, the invention can comprise selecting an hydrolase that is effective to induce tolerance in an immune cell to an antigen or cell to which the immune cell was previously reactive. Or, the invention can comprise selecting a hydrolase (or an appropriate mix of hydrolases) that disrupts signal 2 mediators of an immune cell or signal 1 mediators (or both). In one preferred embodiment, the invention can comprise selecting a hydrolase (or an appropriate mixture of hydrolases) that disrupts signal 2 mediators, while leaving in place signal 1. Alternatively, the invention can comprise selecting a hydrolase that removes, destroys, inactivates or disables at least 60% of the cell surface adhesion molecules involved in mediating signal 2 for which a purified krill-derived multifunctional enzyme ("PHM protease," as described, for example, in U.S. Patents 5,945,102 and 5,958,406) or cod trypsin removes, destroys, inactivates or disables at least 60%. Preferably, the amount of such cell surface adhesion molecules removed, destroyed, inactivated or disabled is an amount greater than or within 10% of the amount removed, destroyed, inactivated or disabled by PHM or cod trypsin. The method can comprise treating the donor tissue *ex vivo*.

In one embodiment, the hydrolase employed is more effective on a molar basis in preventing or ameliorating donor tissue rejection than is the krill multifunctional enzyme. For example, the hydrolase employed is more effective in removing one or more of CD4, CD8, CD25 (IL-2 alpha receptor chain), CD28, ICAM-1 (CD54), CD152

(also known as CTLA-4), GP39 (also known as CD154, CD40 ligand or CD40L), an integrin, CD40 and CD80 (also known as B7) than is the krill multifunctional enzyme. Or the hydrolase is more effective than papain, or bromelain, or mammalian trypsin. For example, the hydrolase employed is more effective in removing one or more of CD28, ICAM-1 (CD54), GP39 (CD154), an integrin, CD40 and CD80 than is the krill multifunctional enzyme. In another example, the hydrolase employed is more effective in removing CD28 than is one or more of the krill multifunctional enzyme. In another example, the hydrolase employed is more effective in removing ICAM-1 (CD54) than is one or more of the krill multifunctional enzyme. In another example, the hydrolase employed is more effective in removing an integrin than is one or more of the krill multifunctional enzyme. In another example, the hydrolase employed is more effective in removing an LFA-1 (also known as αL or CD11a) than is the krill multifunctional enzyme. In another example, the hydrolase employed is more effective in removing GP39 (CD154) than is one or more of the krill multifunctional enzyme.

In one embodiment of the method described above, the preventing or ameliorating transplantation rejection reactions comprises treating a donor source of immune cells (lymphocytes such as T-cells or B-cells) with a rejection preventing or ameliorating effective amount of a hydrolase that is effective to reduce the amount of one or more cell surface adhesion molecules, or preventing or ameliorating transplantation rejection. The method can comprise contacting the treated immune cells, which cells are obtained from a recipient animal, with second cells of a donor animal; and transplanting a tissue from the donor animal to the recipient animal

The invention also provides a method of preventing or ameliorating transplantation rejection reactions comprising: treating a donor source of immune cell (e.g., lymphocyte) *precursor* cells (such as from bone marrow) with a rejection preventing or ameliorating effective amount of a hydrolase, and administering the treated lymphocyte precursor cells to a recipient.

The invention further provides a method of preventing or ameliorating transplantation rejection reactions comprising: isolating from a source of immune cells taken from a donor (a) a fraction enriched in mature T-cells and (b) a fraction containing immune cell precursor cells; treating the mature T-cells of fraction (a) with a rejection preventing or ameliorating effective amount of a hydrolase; and administering the

mature T-cells of fraction (a) and fraction (b) to a recipient. In one embodiment, the hydrolase treated mature T-cells are contacted with cells of fraction (b) prior to administration to the recipient.

The invention still further provides a method of preventing or ameliorating transplantation rejection reactions comprising: treating a source of immune cells taken from a recipient or donor (for example where the recipient does not have an immune system) with a rejection preventing or ameliorating effective amount of a hydrolase; incubating the treated source of immune cells with a donor organ, tissue or cell type; transplanting the donor organ, tissue or cell type into the recipient; and administering the treated cells into the recipient. In one embodiment, the treated cells include mature T-cells.

The invention also provides a method of preventing or ameliorating allergic or autoimmune reactions comprising: treating a source of immune cells taken from a treatment subject or donor (for example where the recipient does not have an immune system) with an allergic or autoimmune reaction preventing or ameliorating effective amount of a hydrolase; exposing the immune cells to an antigen that induces the allergic reaction or which contains autoimmune epitopes; and restoring the treated and exposed immune cells cells to the treatment subject.

In one embodiment, the invention provides a method of preventing or ameliorating allergic, autoimmune or transplantation rejection reactions with a hydrolase, comprising: identifying the hydrolase or mixture of hydrolases as a hydrolase or mixture of hydrolases with a relative selective preference for disabling signal 2 and/or signal 1, or effective for inducing tolerance in immune cells to a substance or to a cell; treating immune cells with the hydrolase or mixture of hydrolases; and administering the treated cells to a mammal. Alternatively, the hydrolase can be selected on the basis of specificity for cell surface adhesion molecules.

The invention further provides (a) methods relating to certain conditions using effective amounts of hydrolase, (b) compositions for use in such methods, (c) pharmaceutical compositions containing effective amounts of hydrolase for use in such methods, and (d) uses of the hydrolase composition for manufacturing a medicament for use in such methods. The methods are include:

- treating a tissue, body fluid or composition of cells to remove or inactivate a cell adhesion component comprising, wherein the enzyme is administered to the tissue, body fluid or composition of cells, preferably a cell-adhesion component removing or inactivating effective amount or an immune rejection inhibiting amount of the enzyme is administered, wherein preferably the tissue, body fluid or composition of cells is treated extra-corporeally, although they may also be treated *in situ* in an animal; or
- treating or prophylactically preventing HIV infection, preferably administering an HIV infection treating or preventing effective amount of the enzyme

The method comprises administering a composition comprising a hydrolase described above.

The invention further provides (a) methods for treating or prophylactically preventing a cell-cell or cell-virus adhesion syndrome comprising administering an anti-adhesion effective amount of a hydrolase effective to remove or inactivate a cellular or viral acceptor or receptor adhesion component that is involved in the cell-cell or cell-virus adhesion, (b) compositions or substances for use in such methods, (c) pharmaceutical compositions containing effective amounts of enzyme for use in such methods, and (d) uses of the enzyme composition for manufacturing a medicament for use in such methods. Preferably, the syndrome comprises inflammation, shock, tumor metastases, autoimmune disease, transplantation rejection reactions or microbial infections. Preferably, (a) the syndrome is selected from the group consisting of graft versus host disease, organ or tissue transplantation rejection, autoimmune disease and associated conditions, microbial infection, immune disorder, cystic fibrosis, COPD, atherosclerosis, cancer, asthma, septic shock, toxic shock syndrome, conjunctivitis, reperfusion injury and pain, and (b) a cell surface adhesion molecule, associated with the cell-cell or cell-virus adhesion syndrome, is removed or inactivated by the administered hydrolase, where the cell surface adhesion molecule can be selected from the group consisting of ICAM-1 (also know as CD54), ICAM-2 (also known as CD102), VCAM-1, CD3, CD4, CD8, CD11, CD18, CD28, CD29D, CD31, CD44, CD 49, CD62L, CD102, GP39 (CD154), integrins (e.g., of β-1 subfamily {e.g., β-1 (CD29) with α1 (CD49a), α2 (CD49b), α3 (CD49c), α4 (CD49d), α5 (CD49e), α6 (CD49f) or αV (CD51), of β-2 subfamily (e.g., β-2 (CD11a) with αL (CD11b), αM (CD) or αX (CD11c), or of β-3

subfamily (e.g., β-3 (CD61) with αV (CD51) or α11b (CD41), β-4 (CD104) with α6 (CD49f), β-5 with αV (CD51), β-P with α4 (CD49d)} and asialo GM1 ceramide.

The invention further provides a pharmaceutical composition for removing or inactivating a cell-surface adhesion molecule comprising a cell-surface adhesion molecule removing or inactivating effective amount of a hydrolase. Such hydrolases include a number of enzymes such as cod trypsin and other hydrolases, including, as one specific example, proteases with multiple classes of proteolytic activity such as the multifunctional enzyme having: activity comprising at least one of a chymotrypsin, trypsin, collagenase, elastase or exo peptidase activity; a molecular weight between about 20 kd and about 40 kd as determined by SDS PAGE; and substantial homology to the krill-derived multifunctional hydrolase. Such compositions typically include a pharmaceutically acceptable diluent or carrier.

The invention still further provides a pharmaceutical composition for treating or prophylactically preventing a cell-cell or cell-virus adhesion syndrome comprising a cell-cell or cell-virus adhesion syndrome treating or preventing effective amount of a composition comprising a hydrolase. For example, in some embodiments the hydrolase is multifunctional enzyme having: activity comprising at least one of a chymotrypsin, trypsin, collagenase, elastase or exo peptidase activity; a molecular weight between about 20 kd and about 40 kd as determined by SDS PAGE; and substantial homology to the krill-derived multifunctional hydrolase. Such compositions typically include a pharmaceutically acceptable diluent or carrier.

In a preferred embodiment, HIV-infected patients are treated to slow the progression of the associated diseases by the process of (1) isolating T-cells from the patient, (2) treating the T-cells with a hydrolase effective to remove CD4, and (3) injecting the T-cells into the patient.

In one aspect, the method of extra-corporeally treating a tissue, body fluid or composition of cells to remove cell adhesion components reduces the immune rejection of a tissue, body fluid or composition of cells that is transplanted from one individual to another. In another aspect, such treatments remove or inactivate the cell adhesion components found in the treated tissue, body fluid or composition of cells involved in a microbial infection.

In some specific embodiments, the invention relates to a hydrolase having multifunctional activity comprising at least one of a chymotrypsin, trypsin, collagenase, elastase or exo peptidase activity, a molecular weight between about 20 kd and about 40 kd as determined by SDS PAGE, and substantial homology to krill-derived multifunctional hydrolase. Preferably, the enzyme has a molecular weight of from about 26 kd to about 32 kd as determined by SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis ("PAGE"), more preferably about 29 kd. Preferably, the enzyme has an N-terminal sequence comprising: I-V-G-G-X-E/D-B-X-X-X-X-Z/B′-P-Z/H-Q-B-X-B′/Z, wherein X is any amino acid, Z is an aromatic amino acid, B is an amino acid having a C1 to C6 alkyl side chain, and B′ is leucine or isoleucine. More preferably, all amino acids represented by X, Z or B are natural amino acids. Preferably, the enzyme has an N-terminal sequence comprising: I-V-G-G-X-E/D-B wherein X is any amino acid, B is an amino acid having a C1 to C6 alkyl side chain. Preferably, the enzyme is the krill-derived multifunctional hydrolase. Thus, in one embodiment, the N-terminal sequence is I-V-G-G-X-E-V-T-P-H-A-Y-P-W-Q-V-G-L-F-I-D-D-M-Y-F (SEQ ID NO. 20). Preferably, the enzyme has the N-terminal sequence: I-V-G-G-N/M-E-V-T-P-H-A-Y-P-W-Q-V-G-L-F-I-D-D-M-Y-F (SEQ ID NO. 1).

In these specific embodiments, preferably, the multifunctional enzyme of the invention has at least two of the identified proteolytic activities, more preferably at least three, still more preferably at least four. Yet more preferably, the enzyme has all of the identified proteolytic activities. Preferably, the multifunctional enzyme has substantial anti cell-cell and cell-virus adhesion activity. Preferably, the multifunctional enzyme has substantial homology with the krill-derived multifunctional hydrolase.

In another aspect of this specific embodiment, the multifunctional enzyme shall include an amino acid sequence having at least about 70% identity with a "reference sequence" described below, more preferably at least about 80% identity, still more preferably at least about 90% identity, yet still more preferably at least about 95% identity. The krill-derived multifunctional hydrolase can be the multifunctional enzyme. The reference sequence is (i) the amino acid 64-300 sequence of SEQ ID NO:21, or (i) a sequence which is that of the amino acid 64-300 sequence of SEQ ID NO:21 except that it has

one or more of the amino acid substitutions found in the amino acid 1-185 sequence of SEQ ID NO:22,

one or more of the amino acid substitutions found in the amino acid 72-178 sequence of SEQ ID NOS:23 or 24,

one or more of the amino acid substitutions found in the amino acid 1-211 sequence of SEQ ID NO:25,

one or more of the amino acid substitutions found in the amino acid 66-302 sequence of SEQ ID NO:26, or

has asparagine or lysine at a residue corresponding to residue 68 of SEQ ID NO:21,

wherein identity is calculated by (a) aligning the sequences as described below and determining, over the entire length corresponding to the reference sequence, the average number of substitutions, deletions or insertions for every 100 amino acids of the reference sequence, with this number corresponding to percent identity; or (b) the method of Needleman and Wunch, using the parameters set forth in Version 2 of DNASIS.

Preferably, the hydrolase is selectively reactive with cell-surface receptors such as proteins or glycolipids. Preferably, the hydrolase is substantially purified. In some embodiments, the hydrolase has a purity with respect to macromolecules of at least about 90%, more preferably least about 95%, more preferably about 97%, still more preferably about 99%, yet more preferably 99.7% with respect to macromolecules. For the purposes of this application, "substantially pure" shall mean about 60% purity.

The invention also provides a pharmaceutical composition comprising the multifunctional enzyme of claim 1 and a pharmaceutically acceptable diluent or carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B: Survival of (C57BL/6 x DBA/2)BDFI recipients of semi-allogeneic C57BL/6 bone marrow cells mixed with PHM-treated C57BL/6 splenocytes.

Figure 2: Survival of (C57BL/6 x DBA/2)BDFI recipients of semi-allogeneic C57BL/6 bone marrow cells mixed with protease-treated C57BL/6 splenocytes.

Figure 3 (3A-3C) shows the effects of cod trypsin of PHM incubations on surface markers.

Figures 4 and 5 show the effects of various hydrolases on a number of cell surface adhesion molecules.

DETAILED DESCRIPTION

It has now been established that the multifunctional enzyme and other hydrolases effectively remove or inactivate certain cell-surface adhesion molecules, such as ICAM-1 (CD54), ICAM-2 (CD102), VCAM-1, CD4, CD8, CD28, CD31, CD11a, CD49d, other integrin component chains, CD44, the asialo GM1 ceramide, CD40 and CD80 without affecting cell viability. This adhesion site removal or inactivation phenomenon is believed to provide at least a partial explanation for effectiveness against many, though probably not all, of the indications against which, for example, the multifunctional enzyme is effective.

Again not wishing to be limited by any particular theory, the anti-CD4 cell surface adhesion molecule activity of the multifunctional enzyme is believed to be responsible, at least in part, for the enzyme's HIV-transmission inhibitory activity. The HIV infective pathway utilizes the CD4 cell-surface molecule. *See*, Lentz, "Molecular Interaction of Viruses with Host-Cell Receptors," in *Adhesion Molecules*, Wegner, Ed., Academic Press, 1994, pp. 223-251 at p. 229.

Studies on the destruction or inactivation of cell surface molecules on T-cell exposed to as little as 10 μg/ml of the krill hydrolase for four hours at 37°C have determined that: CD3 and CD90 show little or no change; CD28, CD49, CD29D, CD18 and CD11 are significantly destroyed or inactivated, about 25% to about 40% reduction detectable antigen; ICAM-1 (CD54), ICAM-2 (CD102), CD44, CD31, CD62L (L-selectin), CD4, and CD8 are substantially destroyed or inactivated, generally about 70% to about 100% reduction in detectable antigen. Additionally, antibodies against asialo GM-1 have indicated reductions in the immunologically detectable amount of this ceramide in the membranes of lung epithelial cells following exposure to the multifunctional enzyme of the invention. Further, such treatment of lung epithelial cells with the krill hydrolase reduces the level attachment of *Pseudomonas* bacteria to the lung epithelial cells.

Further studies have established that hydrolases of interest remove certain cell surface molecules that are believed to contribute to the signal 2 pathway for activation of T-cells, these cell surface molecules include one or more of CD4, CD8, CD28, and CD154, while having substantially less effect on the T-cell receptor (TcR), which is involved in the signal 1 pathway for activation of T-cells. While not wishing to be

limited to theory, it is believed that hydrolases that substantially interfere with the signal 2 pathway or another accessory pathway (e.g., remove, destroy, inactivate or disable at least 60% of at least one CD4, CD8, CD28, or CD154) but do not substantially interfere with the signal 1 pathway (e.g., no more than 50% of TcR is removed, destroyed, inactivated or disabled) are effective in the immune rejection embodiments of the invention. It is believed that, for immune cells subjected to such differential disruption in pathways, exposure to certain substances initiates processes that lead to tolerance against immune reactions to those substances. *See*, e.g., Kuby, *Immunology*, Third Edition, W.H. Freeman & Co. 1997; Waldmann, "Transplantation Tolerance — Where Do We Stand," *Nature Med.* 5(11): 1245-1248, 1999. Prior work has indicated that intervention in signal 2, while signal 1 is activated, drives T-cells into an anergic state. The present inventors a shown that such selective disabling of signaling molecules can be done with hydrolases selected to have the appropriate selectivity in removing, destroying, inactivating or disabling cell surface molecules.

It is believed that the above discussed adhesion molecules and others will prove to play a role in a number of other diseases for which the multifunctional enzyme is an effective treatment or preventative agent. As described further in Example 4, it has now been shown that treatments with hydrolases are effective to treat, prevent or reduce the severity of GVHD.

For the purposes of this application, the terms listed below shall have the following meaning:

• **adhesion molecule:** a molecule found on the surface of a cell involved, directly or indirectly, in transmitting signals to the cell.

• **cell-cell or cell-virus adhesion syndrome:** a disease in which a receptor or acceptor cell adhesion component plays a role in the etiology of the disease, for instance by playing a role in the development, transmission, growth or course of the disease.

• **hydrolase:** an enzyme that degrades bonds formed by dehydration reactions such as amide, ester, or ether bonds, The term encompasses, but is not limited to, proteases such as trypsin and chymotrypsin.

• **identity:** "Identity," as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as the case may be, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence

relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" can be readily calculated by known methods, including but not limited to those described in (Computational Molecular Biology, Lesk, A.M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48: 1073 (1988). Preferred methods to determine identity are designed to give the largest match between the sequences tested. Thus, a contiguous portion of a polypeptide can be tested against the reference sequence described above and aligned to give the highest match taking into account that non-matched pairs and non-matched gap sequences are scored against identity, with the each non-matched pairing scoring and each non-matched gap residue or nucleotide reducing the identity, prior to normalization to a percent scale, by -1.

Thus, one of the simplest ways to describe polypeptide sequences that are related, as by high identity, is set forth below for a 95% identity example. In this case the test sequence includes a contiguous segment that is the reference amino acid sequence described above , or is identical with the reference sequence except that, over the entire length corresponding to the reference sequence, the amino acid sequence has an average of up to five substitutions, deletions or insertions for every 100 amino acids of the reference sequence.

Moreover, methods to determine identity are codified in publicly available computer programs. Computer program methods to determine identity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA (Altschul, S.F. et al., J. Molec. Biol. 215: 403-410 (1990). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, MD 20894; Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990). The method of Needleman and Wunch, using the parameters set forth in Version 2 of DNASIS can also be used. Additionally, the well known Smith Waterman algorithm can be used to determine identity.

Alternatively, Parameters for polypeptide sequence comparison include the following:

- Algorithm: Needleman and Wunsch, J. Mol Biol. 48: 443-453 (1970);
- Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff, Proc. Natl. Acad. Sci. USA. 89:10915-10919 (1992);
- Gap Penalty: 12; and
- Gap Length Penalty: 4

A program useful with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison WI. The aforementioned parameters are the default parameters for peptide comparisons (along with no penalty for end gaps).

By way of example, a polypeptide sequence of the present invention may be identical to the reference sequence, that is it may be 100% identical, or it may include up to a certain integer number of amino acid alterations as compared to the reference sequence such that the percent identity is less than 100% identity. Such alterations are selected from the group consisting of at least one amino acid deletion, substitution, including conservative and non-conservative substitution, or insertion, and wherein said alterations may occur at the amino- or carboxy-terminal positions of the reference polypeptide sequence or anywhere between those terminal positions, interspersed either individually among the amino acids in the reference sequence or in one or more contiguous groups within the reference sequence.

• **immune disorder:** any disorder caused by an immune reaction to foreign substances, tissues or cells or to autologous or transplanted tissue. The term encompasses autoimmune diseases.

• **immune cell:** a lymphocyte, such as a B-cell or T-cell, or a precursor cell to a lymphocyte.

• **krill-derived multifunctional hydrolase:** a multifunctional enzyme having the same sequence as the enzyme isolated from krill having the properties of the protein described in Examples 1B, 1C and 1D. This enzyme is also referred to as the "krill multifunctional hydrolase" or the "krill multifunctional enzyme" or the "krill-derived multifunctional enzyme."

• **macromolecule:** for determining purity, this means a biological polymer such as a protein, nucleic acid or carbohydrate of molecular weight greater than about 1000.

• **multifunctional enzyme:** an enzyme having activity comprising at least one of a chymotrypsin, trypsin, collagenase, elastase or exo peptidase activity, a molecular weight between about 20 kd and about 40 kd, and substantial homology to krill-derived multifunctional hydrolase.

• **multifunctional enzyme derived from fish or crustacean:** refers to an enzyme having the same sequence as an enzyme isolated from fish or crustacean.

• **protein:** for the purpose of determining purity, this means a polypeptide of molecular weight greater than about 1000.

• **reactive with a cell-surface protein or glycolipid:** means removes, destroys, inactivates or disables the detectable presence of the cell-surface molecule, by whatever mechanism.

• **reactive with a cellular or viral acceptor or receptor adhesion component:** means removes, destroys, inactivates or disables a cell's or a virus' ability to interact with a cell, virus, ligand, group or molecule, regardless of the mechanism.

• **SDS-PAGE:** means polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulfate.

• **selectively reactive with a cell-surface protein:** means removes, destroys, inactivates or disables certain cell-surface proteins on the surface of a cell but not others.

• **substantial homology:** at least about 60% sequence homology.

• **systemic administration:** an administration of a biological agent, such as the multifunctional enzyme, designed deliver the agent to the blood or other circulatory system (such as the lymphatic system) of an animal.

• **tolerance:** a state of unresponsiveness of an immune cell upon encountering an antigen or cell.

• **units of activity:** Hydrolases have unit activity according to a recognized assay for the particular type of hydrolase, and is typically defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of substrate per minute at 25°C. For the chymotrypsin activity of a hydrolase, succinyl-ala-ala-pro-phe-*p*-nitroanilide (Sigma Chemical Co., St. Louis, MO) is the substrate, and hydrolysis is monitored via the absorbance change at 410 nm. The extinction coefficient, ε, of *p*-nitroanilide is 8800 $M^{-1}cm^{-1}$, thus the multiplication factor to convert dA/minute into U/minute of sample is

5.68, when 20 μl of sample is used. For the trypsin activity of a hydrolase, the substrate is CBZ-GRPpNA.

When HL60 cells (promyelocyte cells believed to give rise to T-cells, derived from an acute progranulocytic leukemia) are pretreated with the krill multifunctional hydrolase, their binding to TNFα stimulated endothelial cells is inhibited by more than about 60%. Preferably, treatment of HL60 or endothelial cells with the multifunctional enzyme of the invention will inhibit HL60 cell binding to TNFα stimulated endothelial cells by at least about 20%, more preferably at least about 40%, still more preferably at least about 60%, yet more preferably at least about 80%. Alternately, the multifunctional enzyme will preferably have at least about 30% of the adhesion-inhibiting activity of the krill-derived multifunctional hydrolase. More preferably, the multifunctional enzyme shall have at least about 60% of the adhesion inhibiting activity of the krill-derived multifunctional hydrolase, still more preferably at least about 80%, yet more preferably at least about 100%.

Transplantation; Autoimmune and Allergic Reactions

Studies on bone marrow transplantation provide an illustration of the effect of hydrolase treatments in treating, inhibiting or preventing an immune rejection, in this case GVHD. GVHD typically involves the donor cells attacking the host, instead of the host attacking the donor tissue. Bone marrow transplantation (BMT) is used in conjunction with treatments of a number of cancers, particularly treatments that damage or destroy cell types found in blood, such as treatments of life-threatening hematologic malignancies. However, the threat of severe graft-vs.-host disease (GVHD) remains a major obstacle, impeding widespread application of bone marrow transplantation. Acute and chronic GVHD develops in a significant proportion of transplant recipients and represents a major cause of morbidity and mortality after bone marrow transplantation between imperfectly matched individuals (i.e., allogeneic transplantation). Efforts to prevent GVHD should reduce morbidity and mortality of transplantation, and enhance the long term outcome of a transplant. GVHD is a T-cell mediated disease affecting multiple organ systems. The risk of death due to GVHD can be reduced by depleting the T-cell population in the marrow inoculum used in bone marrow transplantation, or by using immunosuppressive drugs, such as FK506 or rapamycin (see for review, Blazar et al., 1997). Others have shown that a short course of high dose IL-2 administered at the

time of bone marrow transplantation can protect against GVHD mortality in mice (Sykes et al., 1990; Abraham et al,, 1992). Treatment with a protective course of IL-12 also inhibit GVHD, as IL-12 reduces the kinetics of T-cell expansion (Sykes et al., 1995). In most of these strategies, however, extensive treatments ancillary to transplantation are necessary, and can lead to adverse consequences.

Recent strategies against GVHD have evolved around the concept of inducing immune tolerance in T-cells. In the late 1980's, Jenkins and Schwartz demonstrated that to get full activation, T-cells must receive two signals: one through the T cell receptor (TcR), and a second signal delivered by accessory molecules, such as CD28, which bind to their counter receptors expressed at the surface of antigen presenting cells (APC) (reviewed in Schwartz et al., 1997). Activation of T cells through the TcR in absence of the second signal not only fails to activate T cells, but to the contrary induces a state of unresponsiveness (i.e., anergy). Close interactions between cells also play a crucial role in allorecognition as such interactions facilitate the binding of the TcR to the allo-MHC, and of the accessory receptor to its counter ligand. Indeed, integrins like LFA-1 (αL or CD11a, which associates with CD18) expressed on T cells bind to a counter ligand (ICAM-1, i.e., CD54) on the antigen presenting cell to increase the avidity of the interaction between a T-cell the antigen presenting cell (Dustin et al., 1991; St-Pierre et al., 1991). Consistent with this model is the observation that blocking LFA-1/ICAM-1 interactions with antibodies prevents GVHD only partially, but such blockade significantly increases the efficacy of other blocking antibodies specific for other accessory molecules in inducing a state of anergy in T-cells during GVHD (Blazar et al., 1995; <u>Cavazzana-Calvo</u> et al., 1996).

Treatment of immune cells with hydrolases significantly affects key cell surface adhesion molecules implicated in the delivery of activation signals. It has now been found that CD4, CD8, and other cell adhesion molecules, are among the most sensitive cell surface adhesion molecules to proteolysis. *Ex vivo* treatment of donor T-cells with hydrolase prior to engraftment is believed to block these activation signals and significantly reduce the severity of GVHD. The present work reports the results of two series of experiments in which lethal GVHD was prevented by treatment of mature T-cells with hydrolase (*see*, Example 4). In one experiment, both the krill multifunctional enzyme and a Cod-derived trypsin were effective. In the other experiment, the Cod

trypsin was more effective, probably reflecting the faster digestion kinetics observed with this enzyme.

It is important to note that the protection induced by *ex vivo* treatment of splenocytes with hydrolases was obtained by treating spleen cells of the donor. Hydrolase treatment of spleen cells is believed (without limitation to theory) to prevent full activation of allogeneic T-cells, inducing a state of tolerance that is transferred to bone marrow T-cells and their precursors through the immune mechanism known as "infectious tolerance" (Cobbold and Waldmann, 1998). Thus, the results reported herein have significant impact not only in GVHD resulting from bone marrow transplant, but on solid organ transplantation as well. *Ex vivo* treatment of recipients T-cells with hydrolase, followed by exposure to allogeneic donor MHC, is believed to induce a state of tolerance in these T-cells that is propagated systemically upon re-injection into the recipient. In some embodiments, exposure to donor MHC is conducted *in vitro* (i.e., also *ex. vivo*).

Without limitation to theory, it is believed that the transplantation rejection inhibition seen with the present invention can be explained if the hydrolase-treated immune-mediating cells, when brought into contact with the cells or substances which would trigger immune responses, instead begin the process of acquiring tolerance for such cells or substances. When treated cells are reintroduced into a recipient, such acquired tolerance is believed to be transmitted to other immune cells.

When immune cells are treated and contacted with other immune reaction mediating cells prior to administration to a patient, such contacting is, for example, conducted under appropriate conditions for maintaining metabolically active immune reaction mediating cells for, for example, from a few minutes to a few hours, preferably from about 1 hour to about 4 hours.

In the invention, immune reaction mediating cells are treated with hydrolase, exposed to a preparation which would trigger the immune response sought to be avoided, and reintroduced into a treatment subject. Such hydrolase treatment is typically *ex vivo*, and the exposure is preferably conducted *ex vivo*. Such *ex vivo* exposing (i.e., contacting) is, for example, conducted under appropriate conditions for maintaining metabolically active immune reaction mediating cells for, for example, from a few minutes to a few hours, preferably from about 1 hour to about 4 hours.

Examples of autoimmune-associated antigen preparations include, without limitation, myelin sheath preparations, myelin basic protein and preparations of one or more types of collagen. Antigen preparations can be used, for example, in the treatment of multiple sclerosis, irritable bowel disease (including Crohn's Disease and ulcerative colitis), pernicious anemia, juvenile onset diabetes, thyroiditis, systemic lupus erythematosis (SLE), scleroderma, polyarteritis nodosa and other vasculitides, myasthenia gravis, motor neuron disease, encephlomyelitis, subacute sclerosing pan-encephalitis, Goodpasture's Syndrome, haemolytic anemia, thrombocytopenia, pemphigus vulgaris and bullous pemphigoid. Other examples of autoimmune diseases and examples of allergies can be found in standard texts on allergies or immunology, such as Roitt, *Essential Immunology*, Eighth Edition, Blackwell Scientific Publications, Oxford, 1994.

Transplantation or tolerizing protocols according to the invention include:

1.	Contacting first cells which are immune cells from a recipient animal with second cells or immunogens to which one seeks to induce tolerance, where the first cells or the second cells are immune cells that are treated with hydrolase, administering the first cells to the recipient animal, and, if appropriate, transplanting tissue from the animal source of the second cells to the recipient; and
2.	Contacting first cells which are immune cells from a donor animal with second cells, wherein the first cells or second cells comprise antigens to which one seeks to induce tolerance, where the first cells or the second cells are immune cells that are treated with hydrolase, administering the first cells to a recipient animal, and, if appropriate, transplanting tissue form the animal source of the second cells to the recipient.

The cells contacted with the treated immune cells can be the tissue to be transplanted. Typically, the immune cells are administered some time before transplantation, such as 12, 24, 48, 72 hours. The second cells can also be treated with the hydrolase.

Preferably, the tolerized cells are T-cells. Thus, in one embodiment, the T-cells are tolerized by contact with T-cell depleted cells, preferably immune cells. The T-cells can then be isolated from the tolerizing mixture of cells by an affinity binding protocol or

cell sorting with appropriate cell-specific antibody reagents. For example, Thy-1 (CD90) antibodies tagged with a magnetically susceptible material can be used to isolate T-cells by by magnetic separation.

Exemplary Hydrolases

A wide variety of hydrolases are believed to be applicable. These include metalloproteinases (such as matrix metalloproteinases, including human fibroblast collagenase, interstitial collagenase, stromelysin, gelatinase A, gelatinase B, adamalysins, microbial metalloproteinases and the like), elastases, trypsins, chymotrypsins, other serine proteinases, and the like. Such hydrolases include hydrolases of aquatic origin, as described herein. Other applicable hydrolases are believed to include, for example, mammalian and non-mammalian trypsins, mammalian and non-mammalian chymotrypsins, mammalian and non-mammalian elastases, papains, bromelains, mammalian and non-mammalian collagenases, subtilisins and mammalian and non-mammalian cathepsins (such as cathepsin B, C, D or G). Further enzymes include mixtures of digestive enzymes from Atlantic cod (e.g., trypsin, chymotrypsin, elastase and collagenase), chymotrypsins for Atlantic cod (see, Aseirsson and Bjarnason, *Comp. Biochem. Physiol.* 99B:327-335, 1991; Guthmundsdottir et al., *Biochem. Biophys. Acta.* 1219:211-214, 1994), elastase from Atlantic cod (Aseirsson and Bjarnason, *Biochem. Biophys. Acta.* 1164:91-100, 1993), a mixture of serine proteinase-type collagenases from Atlantic cod (see, Kristjansson et al., *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 110:707-717, 1995), trypsin from Atlantic cod (Aseirsson et al., *Eur. J. Biochem.* 180:85-94, 1989), collagenase from *Uca pugilator* (Tsu et al., *J. Biol. Chem.* 269:19565-19572, 1994), and other hydrolases described herein.

In certain embodiments of the invention, the hydrolase used is not, at least in effective amounts, bromelain, or, in some embodiments, an enzyme component of bromelain. Bromelain is an enzyme-containing extract from pineapple, particularly pineapple stem. Also in certain embodiments, the hydrolase used is not, at least in effective amounts, polymorphonuclear leukocyte-derived proteinase elastase or cathepsin G. Further, in certain embodiments the hydrolase used is not, at least in effective amounts, papain, or mammalian pancreatic trypsin, or a mixture of these enzymes. Papain is an enzyme extracted from papaya.

Signal 2 and Signal 1 Pathways

Preferably, a hydrolase exposed to immune cells removes, destroys, inactivates or disables at least 60%, 75%, 85%, 90%, 95%, 98% or 99% of at least one of CD4, CD8, a CD11, CD25, CD28, a CD49, CD152 and CD154. These molecules are involved in the signal 2 pathway. Preferably, the hydrolases removes, destroys, inactivates or disables a significant portion of (a) CD4 and CD8, (b) CD4 and CD28, (c) CD4 and CD154, (d) CD4, CD8 and CD28, (e) CD4, CD8 and CD154, (f) CD4, CD28 and CD154, (g) CD4, CD8, CD28 and CD154, (h) CD8 and CD28, (i) CD8 and CD154, (j) CD8, CD28 and CD154, or (k) CD28 and CD154. In certain preferred embodiments, the hydrolases removes, destroys, inactivates or disables a significant portion of (a) CD4 and CD8, (d) CD4, CD8 and CD28, (e) CD4, CD8 and CD154 or (g) CD4, CD8, CD28 and CD154.

Preferably, no more than 50%, 35%, 20% or 10% of TcR, which is associated with signal 1, is removed, destroyed, inactivated or disabled by contacting a hydrolase with the immune cells. Preferably, no more than 50%, 35%, 20% or 10% of CD3, which is associated with signal 1, is removed, destroyed, inactivated or disabled by contacting a hydrolase with the immune cells.

Mixtures of Hydrolases

In certain embodiments, a mixture of two or more hydrolases is used to provide the removing, destroying, inactivating or disabling activity, i.e., induce tolerance, or the signal 2 disrupting activity. The mixture can be selected on the basis of mixing a second (or third, etc.) hydrolase that is more effective against given cell surface adhesion molecule than another hydrolase in the mixture.

Co-Administration of Antibodies

In one embodiment of the invention, the effects of hydrolase treatment is supplemented with the use of antibodies to specific cell adhesion molecules. This approach can be used for example to alter the kinetics of cell surface effects or supplement effects against certain cell adhesion molecules. For example, the hydrolase selected could be very effective against certain of the targeted cell surface adhesion molecules, but less effective against others. In this case, the antibodies, which are preferably monoclonal, are used to target the cell surface adhesion molecules against which the hydrolase is less effective. Or, the selected hydrolase can be effective against targeted cell surface adhesion molecules, but a further effect can be achieved with the antibodies. The antibodies can monovalent (e.g., fab fragments), especially as to a given

cell surface target (such that an antibody monovalent as to a cell surface adhesion molecule has another binding pocket with another specificity).

Appropriate targets for antibody blockade include CD4, CD8, CD25 (IL-2 receptor alpha chain), CD28, CD152 (CTLA-4), integrins, CD154, CD40 and CD80.

Antibody sources for use in this aspect of the invention include: Boehringer Mannheim, LaVal, Quebec; GIBCO, New York; PharMingen, San Diego, CA; Wako Bioproducts, Richmond, VA. The antibodies are contacted with the cells in sufficient amounts, and preferably some excess, to bind the available targeted cell surface adhesion molecules.

Administration of Hydrolase

For topical treatments, a suitable dose of hydrolase per application ranges from about 0.1 μg/cm^2 to about 1 mg/cm^2, preferably from about 1 μg/cm^2 (for example, using about 10 μg/ml) to about 1 mg/cm^2 (for example, using about 10 mg/ml), more preferably from about 5 μg/cm^2 (for example, using about 50 μg/ml) to about 100 μg/cm^2 (for example, using about 1 mg/ml), yet more preferably from about 10 μg/cm^2 to about 250 μg/cm^2, still yet more preferably from about 10 μg/cm^2 (for example, using about 100 μg/ml) to about 50 μg/cm^2 (for example, about 500 μg/ml). For systemic treatments, dosages will generally be selected to maintain a serum level of hydrolase between about 0.1 μg/100cc and about 5 μg/100cc, preferably between about 0.5 μg/100cc and about 2.0 μg/100cc. In an alternative measure of preferred systemic administration amounts, preferably from about 0.1 mg/kg to about 10 mg/kg, more preferably about 1mg/kg, will be administered (although toxicology in animal models suggests that amounts even in excess of 25 mg/kg can be used). For ocular treatments, a suitable dose of hydrolase per application ranges from about 0.01 mg per eye to about 5 mg per eye, preferably from about 0.1 mg per eye to about 2.0 mg per eye. For vaginal and urinary tract treatments, suitable flushing/ instillation solutions of the hydrolase will generally have concentrations from about 1 μg/ml to about 15 mg/ml, preferably from about 100 μg/ml to about 3 mg/ml. For oral treatments, suitable mouthwash solutions will generally have concentration of hydrolase from about 1 mg/ml to about 15 mg/ml preferably from about 2 mg/ml to about 10 mg/ml. Lozenges will typically contain from about 100 μg to about 10 mg of hydrolase. Aerosols will generally be made from solutions having enzyme concentrations from about 0.1 mg/ml to about 15 mg/ml,

preferably from about 1 mg/ml to about 10 mg/ml. Generally, from about 0.1 ml to about 2 ml of aerosol will be applied to the airways of the patient, preferably from about 0.5 ml to about 1.0 ml. For scar and keloid treatments, generally between about 0.1 mg and about 5 mg of hydrolase will be injected into each cm^2 of the lesion, preferably from about 0.5 mg to about 3 mg. For treating adhered connective tissue or joints, generally between about 0.5 mg and about 10 mg of hydrolase will be injected interstitially at the adhesion, preferably between about 1 mg and about 5 mg. For all treatments, the enzyme composition will generally be applied from about 1 to about 10 times per day, preferably from about 2 to about 5 times per day. These values, of course, will vary with a number of factors including the type and severity of the disease, and the age, weight and medical condition of the patient, as will be recognized by those of ordinary skill in the medical arts. It is believed that substantially higher doses can be used without substantial adverse effect.

For treating immune disorders, the composition may be applied systemically or in a manner adapted to target the affected tissue or cells, or a tissue or cells implicated in the disorder can be treated extra-corporeally.

For organ transplants or other *ex vivo* treatments, the organ, tissue or cells to be transplanted will preferably be bathed in a solution of the hydrolase for between about 10 minutes and about 5 hours. The enzyme solution will preferably contain between about 0.01 mg/ml or 0.5U/ml and about 25 mg/ml or 1,250U/ml of the hydrolase, and in certain embodiment preferably, between about 0.5 mg/ml or 25U/ml and about 5 mg/ml and about 250U/ml. After transplantation, the hydrolase can be administered systemically using the conditions described above. For treating bone marrow or other sources of cells found in the blood, particularly those containing T-cells or T-cell precursors, the cells are preferably treated with an amount and time of treatment effective to reduce, remove or inactivate at least one cell surface protein by at least about 50%, more preferably by at least about 80%.

The hydrolase of the invention is administered orally, topically, rectally, vaginally, by instillation (for instance into the urinary tract or into fistulas), by pulmonary route by use of an aerosol, by application of drops to the eye, or systemically, such as parenterally, including, for example, intramuscularly, subcutaneously, intraperitoneally, intraarterially or intravenously. The multifunctional enzyme is

administered alone, or it is combined with a pharmaceutically-acceptable carrier or excipient according to standard pharmaceutical practice. For the oral mode of administration, the hydrolase is used in the form of tablets, capsules, lozenges, chewing gum, troches, powders, syrups, elixirs, aqueous solutions and suspensions, and the like. In the case of tablets, carriers that is used include lactose, sodium citrate and salts of phosphoric acid. Various disintegrants such as starch, and lubricating agents such as magnesium stearate and talc, are commonly used in tablets. For oral administration in capsule form, useful diluents are lactose and high molecular weight polyethylene glycols. If desired, certain sweetening and/or flavoring agents are added. For parenteral administration, sterile solutions of the hydrolase are usually prepared, and the pHs of the solutions are suitably adjusted and buffered. For intravenous use, the total concentration of solutes should be controlled to render the preparation isotonic. For ocular administration, ointments or droppable liquids may be delivered by ocular delivery systems known to the art such as applicators or eye droppers. Such compositions can include mucomimetics such as hyaluronic acid, chondroitin sulfate, hydroxypropyl methylcellulose or polyvinyl alcohol, preservatives such as sorbic acid, EDTA or benzylchronium chloride, and the usual quantities of diluents and/or carriers. For pulmonary administration, diluents and/or carriers will be selected to be appropriate to allow the formation of an aerosol. For topical administrations, the hydrolase is typically administered in aqueous form or in a hydrogel. A preferred hydrogel comprises an aqueous suspension of from about 1% (w/v) to about 10% of low molecular weight hydrolyzed starch.

Suppository forms of the hydrolase are useful for vaginal, urethral and rectal administrations. Such suppositories will generally be constructed of a mixture of substances that is solid at room temperature but melts at body temperature. The substances commonly used to create such vehicles include theobroma oil, glycerinated gelatin, hydrogenated vegetable oils, mixtures of polyethylene glycols of various molecular weighty and fatty acid esters of polyethylene glycol. *See*, Remington's Pharmaceutical Sciences, 16th Ed., Mack Publishing, Easton, PA, 1980, pp. 1530-1533 for further discussion of suppository dosage forms. Analogous gels or cremes can be used for vaginal, urethral and rectal administrations.

Numerous administration vehicles will be apparent to those of ordinary skill in the art, including without limitation slow release formulations, liposomal formulations and polymeric matrices.

For adhesion disorders, the cells or viruses involved can include, without limitation, endothelial cells, lymphocytes, including T-cells, tumor cells, microbial cells, viruses, including HIV and herpes. Adhesion processes are believed to be involved in tissue invasion, for instance, by immune cells, microbes, and tumor cells.

Preferred hydrolases are proteases. Particularly preferred is the multifunctional enzyme of the invention.

Generally, the hydrolase will be administered in an effective amount. An effective amount is an amount effective to either (1) reduce the symptoms of the disease sought to be treated, (2) induce a pharmacological change relevant to treating the disease sought to be treated, (3) inhibit or prevent infection or re-infection by an infective agent, or (4) prevent or minimize the occurrence of a non-infectious disease (for instance a disease treatable by blocking a cell adhesion phenomenon).

Humans are the preferred subjects for treatment. However, the hydrolases can be used in many veterinary contexts to treat animals, preferably to treat mammals, as will be recognized by those of ordinary skill in light of the present disclosure.

The adhesion of HL60 cells (a hyman lymphocyte cell line) to endothelial cells is believed to model a mechanism for tumor cell invasion and infection more generally. This adhesion is stimulated by tumor necrosis factor ("TNF ") and inhibited by antibodies to the E-selectin antigen on HL60 cells. E-selectin is a cell surface adhesion protein that appears to bind to a sialated carbohydrate. See, Bevilacqua et al., *Science* (1989) 243:1160.

Preparations of the multifunctional enzyme are active even when not purified to homogeneity. Preparations are described, for example, in WO 96/24371 (Phairson Medical) and WO 98/08863 (Phairson Medical).

Isolations and partial sequences of various fish or crustacean hydrolases have been reported. A number of such reports are identified in Table 1, below.

Table 1 - Sequence Reports

Penaeus vanamelii 1		
	Sequence reported:	Van Wormoudt et al., *Comp Biochem. Physiol.*, 103B: 675-680, 1992 and Sellos and Wormhoudt, *FEBS*, 39: 219-224, 1992.
	Reported activities:	chymotryptic
	Apparent MW:	25kd
Panaeus vanameii 2		
	Sequence reported:	Van Wormoudt et al., *Comp Biochem. Physiol.*, 103B: 675-680, 1992.
	Reported activities	chymotryptic (tryptic)
	Apparent MW:	25kd
Panaeus monodon tryptic (shrimp)		
	Sequence reported:	Lu et al., *Biol. Chem. Hoppe-Seyler*, 371: 851-859, 1990.
	Reported activities:	tryptic
	Apparent MW:	27kd
	Ph optimum:	7.4 - 8.0
	Pi:	2.4
Panaeus monodon chymotryptic - 1 (shrimp)		
	Sequence reported:	Tsai et al., *Biochem et Biophys. Acta*, 1080: 59-67, 1991
	Reported activities:	chymotryptic collagenase
	Apparent MW:	27-28kd
Panaeus monodon chymotryptic - 2		
	Sequence reported:	Tsai et al., *Biochem. et Biophys. Acta*, 1080: 59-67, 1991
	Reported activities:	chymotryptic collagenase
	Apparent MW:	25-26kd
Uca pubilator (Fiddler Crab) 1		
	Sequence reported:	Tsai et al., *Biochem. et Biophys. Acta*, 1080: 59-67, 1991
	Reported activities:	chymotryptic
	Apparent MW: Ph optimum	25kd 8.0 - 8.5

Uca pugilator II		
	Sequence reported:	Grant et al., *Biochemistry*, 19: 4653-4659, 1980.
	Reported activities:	chymotryptic collagenase tryptic elastase
	Apparent MW:	25kd
	pI:	8.0 - 8.5
Kamchatka crab (at least four proteases)		
	Sequence Reported:	Klimova et al., *Biochem. Biophys. Res. Commun.* **166**: 1411-1420, 1990
	Reported Activities:	tryptic collagenase
	Apparent MW:	23-26kd
Crayfish Protease		
	Sequence reported:	Titani et al., *Biochemistry*, 22: 1459-1465,

The sequence of the first 25 amino acids of the Krill derived multifunctional enzyme is I-V-G-G-N/M-E-V-T-P-H-A-Y-P-(W)-Q-V-G-L-F-I-D-D-M-Y-F (SEQ ID NO. 1). The parentheses indicate a weak recovery of the 14th amino acid and "N/M" indicates heterogeneity at the 5th position. A comparison of the N-terminal 20 to 25 amino acid sequences of various serine hydrolases is presented in Table 2, below.

Table 2 - N-Terminal Sequences

Species	SEQ ID NO	Sequence
Penaeus vanameii 1 (shrimp)	3	I V G G V E A T P H S W P H Q A A L F I D D M Y F
Penaeus vanameii 2	4	I V G G V E A T P H S X P H Q A A L F I
P. monodon, trypt. (shrimp)	5	I V G G T A V T P G E F P Y Q L S F Q D S I E G V
P. monodon, chym. 1	6	I V G G V E A V P G V W P Y Q A A L F I I D M Y F
P. monodon, chym. 2	7	I V G G V E A V P H S W P Y Q A A L F I I D M Y F
Uca pugilator I (crab)	8	I V G G V E A V P N S W P H Q A A L F I D D M Y F

Species	SEQ ID NO	Sequence
Uca pugilator II	9	I V G G Q D A T P G Q F P Y Q L S F Q D
King crab	10	I V G G Q E A S P G S W P ? Q V G L F
Kamchatka crab I	11	I V G G Q E A S P G S W P X Q V G L F F
IIA	12	I V G G T E V T P G E I P Y Q L S L Q D
IIB	13	I V G G T E V T P G E I P Y Q L S F Q D
IIC	14	I V G G S E A T S G Q F P Y Q X S F Q D
Crayfish	15	I V G G T D A T L G E F P Y Q L S F Q N
krill Enzyme	1	I V G G N E V T P H A Y P W Q V G L F I D D M Y F
	2	I V G G M E V T P H A Y P W Q V G L F I D D M Y F
Bovine chymotrypsn	16	I V N G E D A V P G S W P W Q V S L Q D
Salmon	17	I V G G Y E C K A Y S Q A Y Q V S L N S G Y H Y C
Atlant. Cod I*	18	I V G G Y E C T K H S Q A H Q V S L N S G Y H
Atlant. Cod II*	19	I V G G Y E C T R H S Q A H Q V S L N S G Y H
Atlant. Cod Tryspin	27	I V G G Y Q C E A H S Q A H Q V S L N S G Y H Y C G G S L I N W V V S A A

*Both of these enzymes are trypsins; see, Gudmundsdottir et al., *Eur. J. Biochem.* **217**: 1091-1097, 1993.
X = unknown or undefined.

It will be apparent to those of ordinary skill that the enzyme can be manufactured by recombinant means. For instance, the sequences recited herein can be used as the basis of oligonucleotide probes for screening expression or genomic libraries to isolate the complete structural gene. *See, e.g.*, Suggs et al., *Proc. Natl. Acad. Sci. USA*, 78: 6613, 1981 or Berent et al., *BioTechniques*, 3: 208, 1985. Alternately, known protein sequences can be used to design primers for use in PCR-based amplification of nucleic acid encoding a multifunctional enzyme. *See generally*, *Molecular Cloning: A Laboratory Manual, second edition*, Cold Spring Harbor, 1989 and *PCR Protocols, A Guide to Methods and Applications*, edited by Michael et al., Academic Press, 1990. Once fully identified, these structural genes can be edited and appropriately inserted into expression vectors by methods known to the art. In particular, recombinant means can follow the guidance found in WO 98/08863 (Phairson Medical).

These structural genes can be altered by mutagenesis methods such as that described by Adelman et al., *DNA*, 2: 183, 1983 or through the use of synthetic nucleic acid strands. The products of mutant genes can be readily tested for multifunctional enzymatic activity. Conservative mutations are preferred. Such conservative mutations include mutations that switch one amino acid for another within one of the following groups:

1. Small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro and Gly;
2. Polar, negatively charged residues and their amides: Asp, Asn, Glu and Gln;
3. Polar, positively charged residues: His, Arg and Lys;
4. Large aliphatic, nonpolar residues: Met, Leu, Ile, Val and Cys; and
5. Aromatic residues: Phe, Tyr and Trp.

A preferred listing of conservative substitutions is the following:

Original Residue	**Substitution**
Ala	Gly, Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Ala, Pro
His	Asn, Gln
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Tyr, Ile
Phe	Met, Leu, Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp, Phe
Val	Ile, Leu

The types of substitutions selected can be based on the analysis of the frequencies of amino acid substitutions between homologous proteins of different species developed by Schulz et al., *Principles of Protein Structure*, Springer-Verlag, 1978, pp. 14-16, on the analyses of structure-forming potentials developed by Chou and Fasman, *Biochemistry*

13, 211, 1974 or other such methods reviewed by Schulz et al, *Principles in Protein Structure*, Springer-Verlag, 1978, pp. 108-130, and on the analysis of hydrophobicity patterns in proteins developed by Kyte and Doolittle, *J. Mol. Biol.* 157: 105-132, 1982.

Krill, including without limitation krill of the genuses *Euphausia* (such as superba, crystallorphias, frigida, triacantha, vellantini, lougirostris, lucens, similis, spinifera, recurva and the like), *Meganyctiphanes* (such as norvegica and the like) and *Tysanoessa* (such as macurura, vicina, gregaria and the like), are a preferred source of the multifunctional enzyme.

<u>Example 1 - In Vitro Binding of HL60 Cells to Endothelial Cells</u>

Endothelial cells were first passaged onto 96 well plates at a given concentration. The endothelial cells used in the experiment are described in Edgell et al., *Proc. Natl. Acad. Sci. USA* (1983) 80:3734. The cells were incubated at 37°C under a DMEM cell culture medium containing 10% fetal calf serum and under a 5% CO_2 atmosphere. Then, the medium was removed and replaced with 100 µl (microliter) of a suspension of 200,000 HL60 cells (a human lymphocyte cell line, available from the European Cell Culture Bank under ECACC Accession No. 85011431) in RPMI medium containing 10% fetal calf serum. The cells were incubated for 30 minutes. After this, the medium was removed and the adherent cells were washed two times with DMEM medium. The relative adherence of the HL60 cells was measured by measuring the difference in optical density at 450nm between the plates on which the cells were co-incubated and plates having endothelial cells alone.

The effect of TNFα was measured by adding TNFα at 1500 units/ml to the endothelial cells 4 hours before the incubation with HL60 cells. The effect of antibody to E-selectin was measured by adding 25µg/ml of monoclonal antibody BBAZ (R&D Systems Europe, Oxford, England) to the HL60 cells. The results of the experiments were:

Expt. No.	HL60 Cells	Endothelial Cells	Absorbance*
1	no treatment	no treatment	0.324
2	no treatment	pretreated with TNFα	0.444
3	added in the presence of mAb to E-selectin	pretreated with TNFα	0.357

*increase over absorbance of endothelial cells alone

The effects of the krill multifunctional hydrolase on this system were measured by:

1. measuring the effect of adding to the endothelial cells 92.3μg/ml krill multifunctional hydrolase (prepared as in Example 1C of WO 96/24371 (Phairson Medical)) together with the HL60 cells;
2. after pretreating the endothelial cells with TNF for 2 hours, adding 92.3 μg/ml krill multifunctional hydrolase and incubating for 2 more hours prior to the addition of HL60 cells; or
3. pretreating the HL60 cells with 92.3 μg/ml krill multifunctional hydrolase prior to adding the HL60 cells to the plates of endothelial cells.

The results of these experiments were as follows:

Expt. No.	HL60 Cells	Endoth Cells	Absorb-ance*
4	Multifunctional enzyme added simultaneously with cells	pretreated with TNFα	0.425
5	no treatment	Four hours pretreatment: 0-4h TNFα 2-4h multifunctional enzyme	0.247
6	pretreated with multifunctional enzyme for 2h	pretreated with TNFα	0.160
7	pretreated with multifunctional enzyme for 2h	Four hours pretreatment: 0-4h TNFα 2-4h multifunctional enzyme	0.059

*increase over absorbance of endothelial cells alone.

To confirm these results, the number of adhering HL60 cells were counted by removing them from the plate and counting the cells. The number of HL60 cells was determined by subtracting the cell numbers for control plates having only endothelial cells. These counting results mirrored the optical density results, as follows:

EXPERIMENT	HL60 CELL NUMBER
1	32,590
2	43,990
3	35,730
4	42,190
5	25,280
6	17,010

These adherence studies show that krill hydrolase destroyed the cell-surface ligand and acceptor molecules that facilitate cell-adhesion.

Example 2- Activity Against Certain Cell-Surface Adhesion Molecules

Freshly isolated T-cells from the thymus of a C57BL/6 mouse were washed three times with serum-free medium. 1 ml aliquots of the cells containing 5 - 10 X 10^6 cells were treated at 37°C for 4 hours with 0, 100 or 500 µg/ml of the krill-derived multifunctional hydrolase prepared according to Example 1B dissolved in serum-free medium. Resulting cells were labeled with one of fluorescent antibodies identified below:

Antibody	Source
CD4-PE	Boehringer Mannheim, Laval, Quebec
CD8-Red613	GIBCO, Long Island, New York
ICAM-1	PharMingen, San Diego, CA
ICAM-2	PharMingen, San Diego, CA
CD44	PharMingen, San Diego, CA
H-2K	PharMingen, San Diego, CA

The amount of antibody binding was determined using a fluorescence-activated cell sorter. From the results, it was determined that the order of sensitivity to inactivation or removal by the hydrolase was CD4, CD8 < ICAM-2 (CD102) < CD44 < ICAM-1 (CD54) < H-2K. Using these same methods with appropriate cells, including endothelial cells, including the s-end-1 endothelial cell line (Kinashi et al., *J. Leukocyte Biol.* 57: 168, 1995) and T-cells isolated from the thymuses of C57BL/6 mice, it was determined

that the VCAM-1, CD28, CD 31 and asialo GM1 ceramide markers are sensitive to the hydrolase. The antibodies used to make these determinations were:

Antibody Specificity	Source
VCAM-1	PharMingen, San Diego, CA
CD28	PharMingen, San Diego, CA
CD31	PharMingen, San Diego, CA
asialoGM1	Wako Bioproducts, Richmond, VA

In some cases, binding was detected with a labeled second antibody, for instance, binding of the asialo GM1 antibody was detected with FITC-labeled Fab fragments that were specific for rabbit IgG (heavy and light chains), which was obtained from Caltag Laboratories, San Francisco, CA.

Example 3 - Timecourse of Cell Surface Recovery of Adhesion Molecules

O-11.10 T-cell hybrids (this cell line is described by Shimonkevitz et al., *J. Experimental Med.* 158: 303, 1983) were treated with 500 µg/ml of the krill-derived multifunctional hydrolase prepared as described in Example 1B of WO 96/24371 (Phairson Medical) and tested for the CD4 marker as described in Example 2. Immediately after the treatment, well less than 1% of the amount of CD4 found in the controls was found on the hydrolase-treated cells. 48 hours later, the levels in treated cells were the same as those in untreated cells.

Example 4 - GVHD and Bone Marrow Transplantation

Materials and Methods

Mice

Female C57BL/6 (H-2^h), DBA/2 (H-2^k), and (C57BL/6 x DBA/2)F_1 mice (abbreviated BDF1 mice, H-$2^{h,k}$) were purchased from Charles River Laboratories (St-Constant, Quebec, Canada). Animals were housed microisolator cages at the Institut Armaud Frappier specific-pathogen free facility. At the time of bone marrow transplantation, donors and recipients were 6 to 8 weeks of age in the first series of experiments, and 5 to 10 weeks of age in the second series of experiments.

Bone marrow transplantation (BMT)

Recipients were given a single dose of 700 r total body irradiation 2-4 hours before transplantation from a ^{60}Co irradiator. Irradiated recipients received, as a source of T-cells, a single intravenous injection via the tail vein of 5 x 10^6 bone marrow cells and 5 x 10^6 spleen cells. Spleens, femurs, and tibias were aseptically collected from

euthanized donors and placed in ice-cold Hank's balanced salt solution (HBSS). Spleens were pressed through sterile wire mesh to obtain single cell suspensions which were further treated with Tris-buffered ammonium chloride or sterile distilled water (hypotonic lysis) to eliminate erythrocytes. Bone marrow cells were flushed with a needle and a syringe from femoral and tibial cavities of donor mice and collected. All cell suspensions were washed twice with HBSS before use.

Treatment of spleen cells with proteases

In some experiments, spleen cells were treated with proteases prior to injection in irradiated recipients. The purified krill-derived multifunctional enzyme ("PHM protease") was obtained from Phairson Medical Ltd (Batch No. PS-3; London, England) in a freeze-dried form and reconstituted with sterile serum-free RPMI medium. Cod trypsin was obtained in a liquid form from Dr. Jon Bjarnason (University of Iceland) (Lot NO. 27.11.95) and had a specific activity of 173 U/mg (CBZ-GRPpNA hydrolyzing activity). The enzyme was dialyzed against 1 L of serum-free RPMI stored in frozen aliquots. Papain was obtained from Sigma (St. Louis, MO). In the first series of experiments, donor C57BL/6 spleen cells were treated with 50 μg/ml of PHM in serum-free RPMI medium for 2 h at 37°C, whereas DBA/2 cells were treated with 20 μg/ml of PHM in serum-free RPMI medium for 2 h at 37°C. In the second series of experiments, donor C57BL/6 spleen cells were treated with 20 μg/ml of protease (e.g. cod trypsin, papain, or PHM) in serum-free RPMI medium for 1 h at 37°C. Controls included spleen cells incubated without proteases in serum-free RPMI for the same period of time at 37°C. Cells were then washed twice in serum-free medium and counted using Trypan blue staining,

Flow cytometry

Spleen cells from C57BL/6 mice were stained with saturating amount of PE-labeled anti-CD4 (Pharmingen, San Diego, CA) and Red-613-labeled ant-CD8 antibodies (GIBCO-BRL, Mississaugua, Ont, Canada) obtained commercially. The stained cells were analyzed on a Coulter XL-MCL laser flow cytometer (Hialeah, FL).

Experimental design

The experiments were designed to investigate the impact of protease treatment of splenocytes on the prevention of lethal GVHD. Donor cells from C57BL/6 (H-2^h), or DBA/2 (H-2^k), were injected in semi-allogeneic BDFI (H-$2^{b,k}$) recipients. In this model,

rejection of the bone marrow graft is not possible as H-2^b or H-2^k cells are recognized as self by the BDF1 recipients. GVHD is induced either upon allorecognition of H-2^k antigens expressed by the antigen presenting cells of the recipients following injection of C57BL/6 (H-2^b) donor T-cells, or upon allorecognition of H-2^b antigens on the antigen presenting cells of the recipients following injection of DBA/2 (H-2^k) donor T-cells. In this model of GVHD, mature donor T-cells are mixed with the bone marrow inoculum since the number of donor T-cells in the marrow inoculum is insufficient to induce reproducible and acute GVHD (Ushiyama et al. 1995). Since all nucleated cells express H-2 antigens, the attack of the donor T-cells can be severe, and kill the animals (acute GVHD). Sometimes depending on the H-2 mismatch between donor and recipients, the GVH reaction is mild, and does not kill the recipients (e.g. chronic GvHD). Mice were observed periodically for clinical signs of the disease, and their weight measured twice a week.

<u>*Results*</u>

The first-series of experiments:

PHM can cleave several cell surface adhesion molecules from the surface of T lymphocyte cell lines in vitro, including CD4, CD8, CD62L, CD54, and others. Thus, PHM can cleave receptors from the surface of freshly isolated splenocytes of C57BL/6. The cleavage of the CD4 and CD8 molecules by PHM was dose-dependent. When PHM is used at concentrations above 20 μg/ml, we found that PHM completely removed the expression of CD4 or CD8 from the surface of splenocytes. Splenocytes from C57BL/6 mice were incubated with the indicated concentrations of PHM for 1 h at 37°C in serum-free RPMI medium. Cells were then stained with specific antibodies to CD4 or CD8, and analyzed by laser flow cytometry.

Since PHM can remove any expression of CD4 or CD8 at the surface of splenocytes, and since both CD4 and CD8 have been reported to play a key role in the induction of GVHD, the question of whether *ex vivo* treatment of lymphoid cells with PHM could reduce the adverse effect of GVHD in a murine model of severe GVHD was investigated. In the first series of experiments, a group (n = 6) of lethally irradiated BDF1 (C57BL/6 x DBA/2, H-$2^{b,k}$) recipients were reconstituted with 5 x10^6 bone marrow and 5 x 10^7 splenocytes from C57BL/6 (H-2^b) mice. In this model, spleen cells were added to the bone marrow inoculum since there is often not enough T-cells in the

bone marrow to induce severe GVHD. Under these conditions, BDF1 recipients reconstituted with C57BL/6 bone marrow cells and splenocytes did not survive allogeneic BMT, as most of recipients died within 4 weeks post-transfer (untreated group, Figure 1A). In contrast, C57BL/6 inoculums of bone marrow cells + splenocytes from did not induce a GVH reaction when inoculated into histocompatible, lethally irradiated C57BL/6 recipients (syngeneic control, Figure 1A). *Ex vivo* treatment of C57BL/6 splenocytes with PHM (50 µg/ml for 2 h at 37°C) was sufficient to prevent, at least partially, the ability of splenocytes to induce lethal GVHD in BDFl recipients. Whereas BDF1 recipients receiving normal C57BL/6 splenocytes died within 4 weeks post-transfer, most of the recipients (4/6) receiving PHM-treated splenocytes mixed with BMC survived up to 60 days post-transfer. In Figure 1, controls included recipients receiving untreated semi-allogeneic splenocytes (untreated) and recipients receiving syngeneic PHM-treated (50 µg/ml for 2 h at 37°C) splenocytes (Syngeneic + PHM.). Figure 1B illustrates mean survival times (MST) of the three different groups of recipients.

GVHD is associated with severe weight loss. BDFl recipients receiving C57BL/6 bone marrow mixed with splenocytes suffered irreversible and severe weight loss while recipients receiving histocompatible bone marrow inoculum occasionally lost some weight shortly after the transfer due to the irradiation, but subsequently showed signs of recovery as indicated by a continuous gain of weight. BDFl recipients reconstituted with C57BL/6 bone marrow cells and the PHM-treated splenocytes also recovered from the initial weight loss associated with the irradiation then underwent a period of gradual weight loss between day 15 and they 30. After day 30, however, these BDFl recipients started to fully recover and most of these recipients survived and gained weight. For these results, weights of individual mice were monitored twice a week for each BDF1 recipient receiving C57BL/6 semi-allogeneic bone marrow cells mixed with (A) untreated splenocytes, (B) PHM-treated splenocytes, or (C) syngeneic, PHM-treated splenocytes.

In murine models of GVHD, it is sometimes difficult to accurately predict the onset of GVHD, irrespective of the level of histocompatibility between donors and recipients. In the above experiments, the combination of C57BL/6 with BDF1 recipients was indeed a good model of GVHD. To obtain a second model of GVH, we also

reconstituted the BDF1 recipients with DBA/2 bone marrow cells mixed with DBA/2 splenocytes with a similar inoculum of bone marrow cells and splenocytes. However, reconstitution of DBF1 recipients with DBA/2 BMC did not lead to an acute and lethal GVHD. Only one BDF1 recipients died after bone marrow graft. Again, in this model, a temporary weight loss was observed shortly (< 10 days), but all recipients survived and showed continuous gain of weight thereafter.

The second series of experiments:

Since CD4 and CD8 play a crucial role as accessory signals in the T-cell response to allogeneic antigens, treatment of splenocytes with PHM could prevent lethal GVHD by removing all cell surface expression of both receptors at the surface of the treated splenocytes prior to the transfer. To gain further insight into this possibility, a second series of experiments were conducted using three proteases: cod trypsin, PHM and papain. Whereas both cod trypsin and PHM can efficiently cleave CD4 and CD8, papain cannot cleave either receptor efficiently.

Lethally irradiated BDF1 recipients were therefore reconstituted as in the first series of experiments, i.e. using 5 $X10^6$ bone marrow and 5 X 10^7 splenocytes. Additional experiments with the spleen of C57BL/6 donor mice showed that efficient cleavage of CD4 and CD8 at their surface could be obtained using a milder treatment of splenocytes with proteases. Thus, the time of incubation with proteases was lowered from 2 h to 1 h, keeping the temperature of incubation at 37°C, and the dose of proteases for *ex vivo* treatment was lowered to 20 μg/ml. Again treatment of splenocytes with a protease significantly reduced the mortality associated with histoincompatible engraftment (Figure 2). At four weeks post-transfer, most of the recipients having received an inoculum of splenocytes treated with a protease had a significantly higher percentage of survival as compared to those reconstituted with untreated splenocytes. The most significant effect was observed with treatment using cod trypsin, as most of the BDF1 mice reconstituted with cod trypsin-treated splenocytes survived the histoincompatible bone marrow graft from C57BL/6 donors. At day 43 post transfer, most of the these BDFI recipients had stabilized their weight, while some show significant gain of weight.

In the current experiments, lethal GVHD has been prevented. Engraftment has been demonstrated, confirming the indication of successful engraftment that follows

from the observation that irradiated recipients that do not receive a bone marrow graft die within one week post-transplantation.

Example 5 - T-Cell Proliferation in Response to Mitogen

Peripheral blood mononuclear cells (PBMCs) were isolated from human blood. 1 X 10^5 PBMCs per well were incubated for 72 h at 37°C in medium supplemented with 10% human serum under 5% CO_2, in the presence of various dilutions of phyto-haemagglutinin (PHA) in the presence (or absence) of a dilution of cod trypsin (diluted 1:100 or more from a 173 U/ml stock solution). At 54 h, the wells were pulsed with ^{3}H-thymidine. After cell harvest, ^{3}H uptake was measured, with each experimental point determined from triplicate cultures. The result was that a dose-responsive diminishment in ^{3}H uptake was seen, with the diminishment first apparent at the 1:1000 dilution and with the 1:100 dilution showing negligible uptake.

A parallel experiment with PHM at best a small effect, but later analysis showed that the starting dilution corresponded to about the 1:10,000 dilution of cod trypsin, such that no effect would have been expected.

Example 6 - T-Cell Proliferation to Alloantigen (Mixed Lymphocyte Reaction)

1 X 10^4 PBMCs (responders) per well were incubated with 1 X 10^5 irradiated allogeneic PBMCs (stimulators). The responder PBMCs and stimulator PBMCs were isolated from the blood of different humans. Cells were incubated under the culture conditions of Example 5 for six days in the presence or absence of cod trypsin (various dilutions). Again a ^{3}H-thymidine pulse was used to generate an uptake indicator of mitogenic activity. The results, from triplicate wells, showed a dose response, with the first significant reduction seen at a 1:10,000 dilution, with the 1:100 dilution showing negligible ^{3}H uptake.

Example 7 - Skin Transplantation in Murine Model

First Protocol

Preparation of Balb/c cells: Spleen cells are harvested, red blood cells removed, and T-cell depleted by magnetic separation with anti-Thy-1 (CD90) antibodies (an antigen specifically expressed on all T cells). T-cell-depleted Balb/c stimulator cells (containing mostly B cells and macrophages, and some dendritic cells, and few NK cells) are resuspended in serum-free RPMI (Russell Park Memorial Institute) at 2 x10^6 cells /ml. PHM or cod trypsin (1, 5, 50 μg/ml final concentration) is added and cells

incubated for 2 hours at 37°C . The reaction with the protease is stopped by adding fetal calf serum (FCS - 10% final concentration). Cells are added to a F-25 flask (10^6 cells/0.5 ml/6.1 cm, i.e. equivalent to 106/24-well plate). An aliquot of cells is used to confirm cleavage by flow cytometric analysis with anti-B7 and anti-CD40 antibodies. Controls include stimulator cells incubated in serum-free medium without PHM.

Preparation of C3H cells: Spleen cells (N.B. 15% of spleen cells are T cells) are harvested as a source of T-cells, red blood cells are removed. Cells are counted and added to F-25 flasks containing PHM-treated C3H stimulator cells at given ratios of 1:1. Cells are incubated for 24 hours at 37°C.

Separation of *ex vivo* tolerized T cells from co-culture: all T-cells from co-culture are isolated using antibodies to Thy-1 (CD90) by magnetic separation (since both CD4 and CD8 are responsible for tolerization (Blazar et al., 1996)). This procedure allows isolation of C3H-T cells away from Balb/c stimulator cells. T-cells are washed twice with PBS to remove any trace of PHM, and the cell concentration adjusted to 108/ml.

Induction of tolerance: Recipient C3H mice are injected iv with 1, 10, and 100 x 10^6 donor cells. Challenge with tail skin from Balb/c mice are done 24, 48 or 72 hours later. Skin transplant, as is known in the art, includes cutting the tails of donor animals, cleaning and trimming the skin (3-4 grafts within a tail), preparing the recipient (anaesthesia), trimming a graft bed, and suturing. Inhibition of rejection is measured 10-12 days post-transplant.

Second Protocol

Preparation of C3H cells: Spleen cells are harvested, red blood cells removed, and T-cell depleted by magnetic separation with anti-Thy-1 (CD90) antibodies (an antigen specifically expressed on all T cells). T-cell-depleted stimulator cells (containing mostly B cells and macrophages, and some dendritic cells, and few NK cells) are resuspended in serum-free RPMI (Russell Park Memorial Institute) at 2 x10^6 cells /ml. PHM or cod trypsin (1, 5, 50 μg/ml final concentration) is added and cells incubated for 2 hours at 37°C. The reaction with the protease is stopped by adding fetal calf serum (FCS - 10% final concentration). Cells are added to a F-25 flask (10^6 cells/0.5 ml/6.1 cm, i.e. equivalent to 106/24-well plate). An aliquot of cells is used to confirm cleavage by flow cytometric analysis with anti-B7 and anti-CD40 antibodies. Controls include stimulator cells incubated in serum-free medium without PHM.

Preparation of Balb/c cells: Spleen cells (N.B. 15% of spleen cells are T cells) are harvested as a source of T-cells, red blood cells are removed. Cells are counted and added to F-25 flasks containing PHM-treated C3H stimulator cells at given ratios of 1:1. Cells are incubated for 24 hours at 37°C.

Separation of *ex vivo* tolerized T cells from co-culture: all T-cells from co-culture are isolated using antibodies to Thy-1 (CD90) by magnetic separation (since both CD4 and CD8 are responsible for tolerization (Blazar et al., 1996)). This procedure allows isolation of Balb/c T-cells away from C3H stimulator cells. T-cells are washed twice with PBS to remove any trace of PHM, and the cell concentration adjusted to 108/ml.

Induction of tolerance: Recipient C3H mice are injected iv with 1, 10, and 100 x 10^6 T-cells. Challenge with tail skin from Balb/c mice are done 24, 48 or 72 hours later. Skin transplant, as is known in the art, includes cutting the tails of donor animals, cleaning and trimming the skin (3-4 grafts within a tail), preparing the recipient (anaesthesia), trimming a graft bed, and suturing. Inhibition of rejection is measured 10-12 days post-transplant.

Example 8 - Effect of Serum

1 X 10^5 PBMCs per well were incubated for 72 h at 37°C in in medium supplemented with 10% human serum under 5% CO_2, in the presence of an appropriate dilution of PHA (selected based on Example 5) in the presence (or absence) of cod trypsin (diluted 1:100) and 0%, 1%, 2%, 5% or 10% human AB serum. The wells were pulsed with ^{3}H-thymidine. After cell harvest, ^{3}H uptake was measured, with each experimental point determined from triplicate cultures. The result was that 1% or 2% serum nearly doubled the hydrolase-induced inhibition of the mitogenic response, while 5% or 10% serum nearly halved the hydrolase-induced inhibition of the mitogenic response.

Example 9 - Removal of Cell-Surface Proteins

Human PBMCs per incubation were incubated at 37°C for 5 h in the presence or absence of 6 μg/ml PHM or 200 μg/ml cod trypsin. The effects of these treatments on various surface markers were measured by flow cytometry with fluorescently labeled antibodies and summarized by percent of cells having the marker and median fluorescence. **Figure 3A** shows the results for CD3, CD4 and CD8, with open squares

representing the results with no enzyme, open triangles for the cod trypsin treatment, and open circles for the PHM treatment. The results for CD25 and CD28 are in **Figure 3B**; for CD11a, CD49a and CD54 in **Figure 3C**.

Example 10 - Protease Comparisons

Aliquots of T-lymphocytes (2 X 10^6) were incubated in 0.5 ml of RPMI 1640 culture medium at 37°C for 2 h in the presence of 2 mcg/ml or 20 mcg/ml of a protease. After incubation, the protease was removed by washing the cells using low speed centrifugation. The effects on surface molecules was quantitated with fluorescent-labeled monoclonal antibodies and fluorescence measured on individual cells by flow cytometry. The quantity is derived from 10,000 cells per measurement. The 2 mcg/ml results are shown in **Figure 4** for CD62L (panel A), CD8 (panel B), CD54 (panel C), CD11a (panel D), CD102 (panel E), CD4 (panel F) and CD31 (panel G). The proteases were:

Enzyme	Source
PHIM	see, U.S. Patents 5,945,102 and 5,958,406
Kamchatka protease	Anawa, Wangen, Switzerland
Cod chymotrypsin	Atlantic cod, see, Aseirsson and Bjarnason, *Comp. Biochem. Physiol.* 99B:327-335, 1991
Cod trypsin	Atlantic cod, see, *European J. Biochem.* 180: 85-94, 1989
Cod collagenase 3	Atlantic cod, see, Aseirsson and Bjarnason, *Comp. Biochem. Physiol.* 99B:327-335, 1991
Cod cryotin IV	Atlantic cod, see, Professor Bjarnason, Univ. or Ra
Cod elastase	Atlantic cod, see, Aseirsson and Bjarnason, *Biochem. Biophys. Acta.* 1164:91-100, 1993
Papain	Sigma Chemical, St. Louis
Bromelain	Sigma Chemical, St. Louis
Subtilisin	Sigma Chemical, St. Louis
Tunisin	Gaiker, Zamudio, Spain
Collagenase F	Sigma Chemical, St. Louis

The 20 mcg/ml results are shown in panels A-G of **Figure 5**.

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The protein sequences described herein and in documents identified herein have been carefully sequenced. However, those of ordinary skill will recognize that nucleic acid sequencing technology can be susceptible to inadvertent error. Those of ordinary skill in the relevant arts are capable of validating or correcting these sequences based on the ample description herein of methods of isolating the nucleic acid sequences in

question, and such modifications that are made readily available by the present disclosure are encompassed by the present invention. Furthermore, those sequences reported herein are believed to define functional biological macromolecules within the invention whether or not later clarifying studies identify sequencing errors. Moreover, please note that sequences recited in the Sequence Listing below as "DNA" or under some other apparently restrictive nomenclature, represent an exemplification of the invention, since those of ordinary skill will recognize that such sequences can be usefully employed in polynucleotides in general, including ribopolynucleotides.

All publications and references, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference in their entirety as if each individual publication or reference were specifically and individually indicated to be incorporated by reference herein as being fully set forth. Any patent application to which this application claims priority is also incorporated by reference herein in its entirety in the manner described above for publications and references.

While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations in the preferred devices and methods may be used and that it is intended that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the claims that follow.

What is claimed:

1. A method of preventing or ameliorating transplantation rejection reactions, where the transplantation can be of immune cells or another tissue, the method comprising:

treating a source of immune cells with a rejection preventing or ameliorating effective amount of a hydrolase or mixture of hydrolases; and

administering the treated immune cells to a recipient animal.

2. The method of preventing or ameliorating transplantation rejection reactions of claim 1, further comprising:

contacting the treated immune cells, which cells are obtained from a recipient animal, with second cells of a donor animal; and

transplanting a tissue from the donor animal to the recipient animal.

3. The method of preventing or ameliorating transplantation rejection reactions of claim 1, further comprising:

administering the immune cells with a cell surface adhesion molecule binding effective amount of an antibody that binds one of CD4, CD8, CD25 (IL-2 receptor alpha chain), CD28, CD152 (CTLA-4), an integrin, CD154, CD40 and CD80.

4. The method of preventing or ameliorating transplantation rejection reactions of claim 1 comprising:

isolating from a source of immune cells taken from a donor (a) a fraction enriched in mature T-cells and (b) a fraction containing immune cell precursor cells;

treating the mature T-cells of fraction (a) with a rejection preventing or ameliorating effective amount of a hydrolase or mixture of hydrolases; and

administering the mature T-cells of fraction (a) and the cells of fraction (b) to a recipient.

5. The method of claim 4, wherein the hydrolase treated mature T-cells are contacted with cells of fraction (b) prior to administration to the recipient.

6. A method of preventing or ameliorating allergic, autoimmune or transplantation rejection reactions comprising:

treating a source of immune cells taken from a recipient with an allergic, autoimmune or transplantation rejection preventing or ameliorating effective amount of a hydrolase or mixture of hydrolases;

contacting the treated source of immune cells with cells from the donor animal or with a substance that induces the allergic reaction or which contains autoimmune epitopes;

transplanting the donor organ into the recipient; and

administering the treated cells into the recipient.

7. The method of claim 6, wherein the treated cells include mature T-cells.

8. The method of claim 6, further comprising

administering the treated immune cells with a cell surface adhesion molecule binding effective amount of an antibody that binds one of CD4, CD8, CD25 (IL-2 receptor alpha chain), CD28, CD152 (CTLA-4), an integrin, CD154, CD40 and CD80.

9. A method of preventing or ameliorating allergic, autoimmune or transplantation rejection reactions with a hydrolase, comprising:

identifying the hydrolase or mixture of hydrolases as a hydrolase or mixture of hydrolases (a) effective to induce tolerance in immune cells to a substance against which the immune cells were previously reactive, or (b) with a relative selective preference for disabling signal 2 and/or signal 1;

treating immune cells or immune cell precursors with the hydrolase or mixture of hydrolases; and

administering the treated cells to a mammal.

10. The method of claim 9, wherein the hydrolase has a relative selective preference for removing, destroying, inactivating or disabling at least one of at least one of CD4, CD8, CD25, CD28, ICAM-1 (CD54), CD152, an integrin, CD154, CD40 and CD80 over removing, destroying, inactivating or disabling TcR.

11. The method of claim 9, wherein the hydrolase has a relative selective preference for removing, destroying, inactivating or disabling at least one of at least one CD4, CD8, CD28, ICAM-1 (CD54), CD11a, CD49d and CD154 over removing, destroying, inactivating or disabling TcR.

12. The method of claim 9, wherein the hydrolase has a relative selective preference for removing, destroying, inactivating or disabling at least two of CD4, CD8, CD28 and CD154 over removing, destroying, inactivating or disabling TcR.

13. A method of identifying a hydrolase for use in preventing or ameliorating allergic, autoimmune or transplantation rejection reactions, comprising:

identifying a relative selective preference of one or more hydrolases for removing, destroying, inactivating or disabling at least one of CD4, CD8, CD25, CD28, ICAM-1 (CD54), CD152, an integrin, CD154, CD40 and CD80 in contrast to removing, destroying, inactivating or disabling TcR; and

selecting a hydrolase or mixture of hydrolases with a relative selective preference for removing, destroying, inactivating or disabling at least one of CD4, CD8, CD25, CD28, ICAM-1 (CD54), CD152, an integrin, CD154, CD40 and CD80 in contrast to removing, destroying, inactivating or disabling TcR.

14. A method of preventing or ameliorating transplantation rejection reactions comprising treating the donor tissue with a rejection reaction preventing or ameliorating effective amount of a hydrolase or mixture of hydrolases, wherein the hydrolase or mixture of hydrolases employed is more effective on a molar basis in preventing or ameliorating donor tissue rejection than is the krill multifunctional enzyme.

15. The method of claim 14, further comprising treating the donor tissue *ex vivo*.

16. The method of claim 14, wherein the hydrolase or mixture of hydrolases employed is more effective in removing, destroying, inactivating or disabling one or more of CD4, CD8, CD25, CD28, ICAM-1 (CD54), CD152, an integrin, CD154, CD40 and CD80 than is the krill multifunctional enzyme.

17. The method of claim 14, wherein the hydrolase or mixture of hydrolases employed is more effective in removing, destroying, inactivating or disabling one or more of CD4, CD8, CD28, ICAM-1 (CD54), an integrin, CD154, than is the krill multifunctional enzyme.

18. A method of preventing or ameliorating transplantation rejection reactions comprising treating a donor source of immune cells with a rejection preventing or ameliorating effective amount of a hydrolase or mixture of hydrolases, wherein the hydrolase or mixture of hydrolases employed is more effective on a molar basis in preventing or ameliorating donor tissue rejection than is the krill multifunctional enzyme.

19. The method of claim 18, wherein the hydrolase or mixture of hydrolases employed is more effective in removing, destroying, inactivating or disabling one or more of CD4, CD8, CD25, CD28, ICAM-1 (CD54), CD152, an integrin and GP39 (CD154) than is the krill multifunctional enzyme.

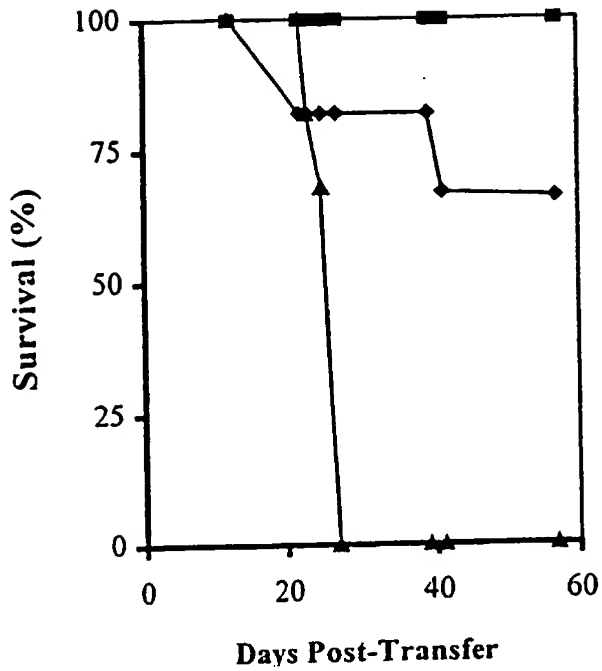


FIG. 1A

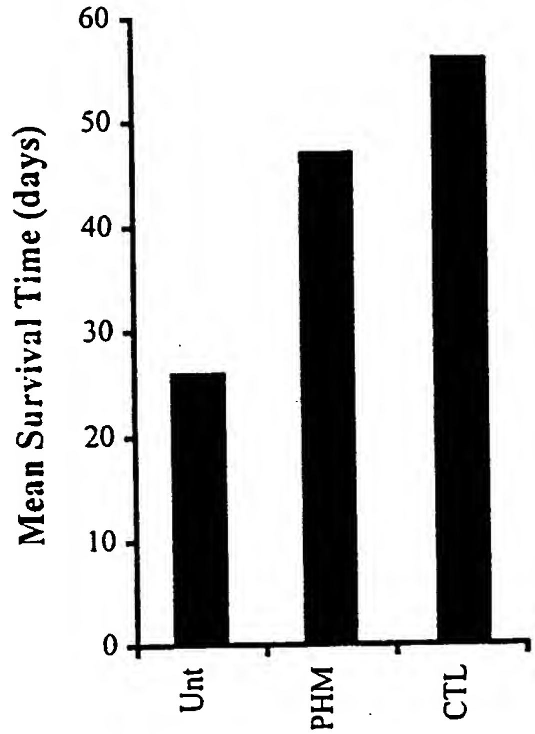


FIG.1B

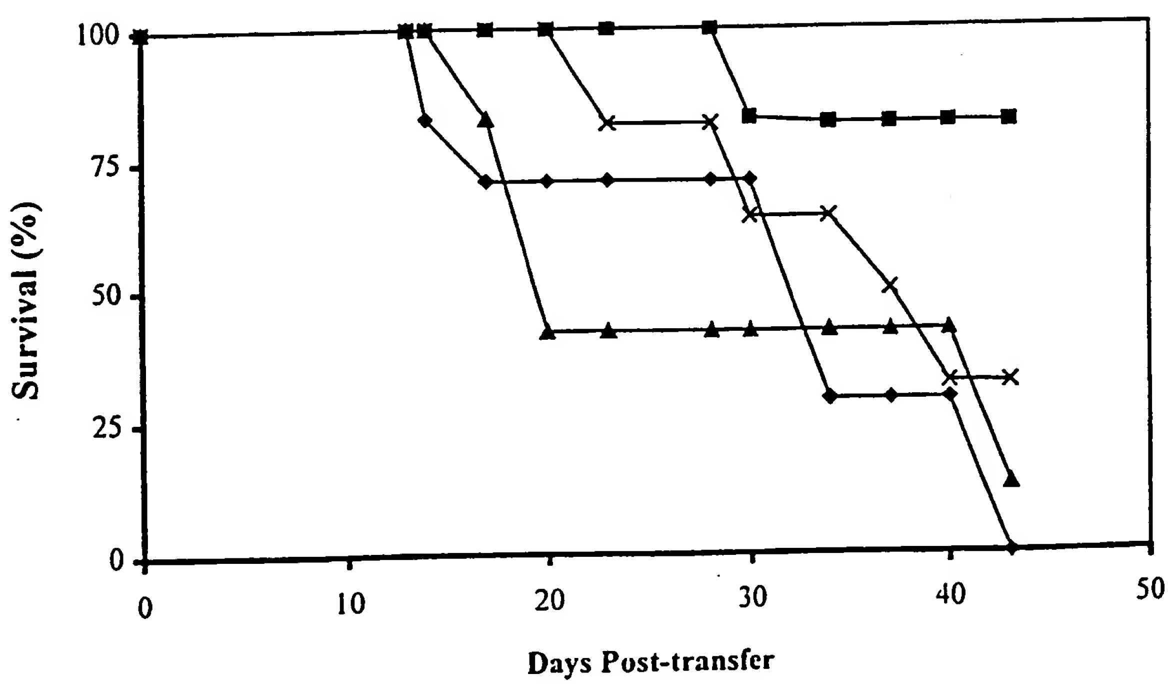
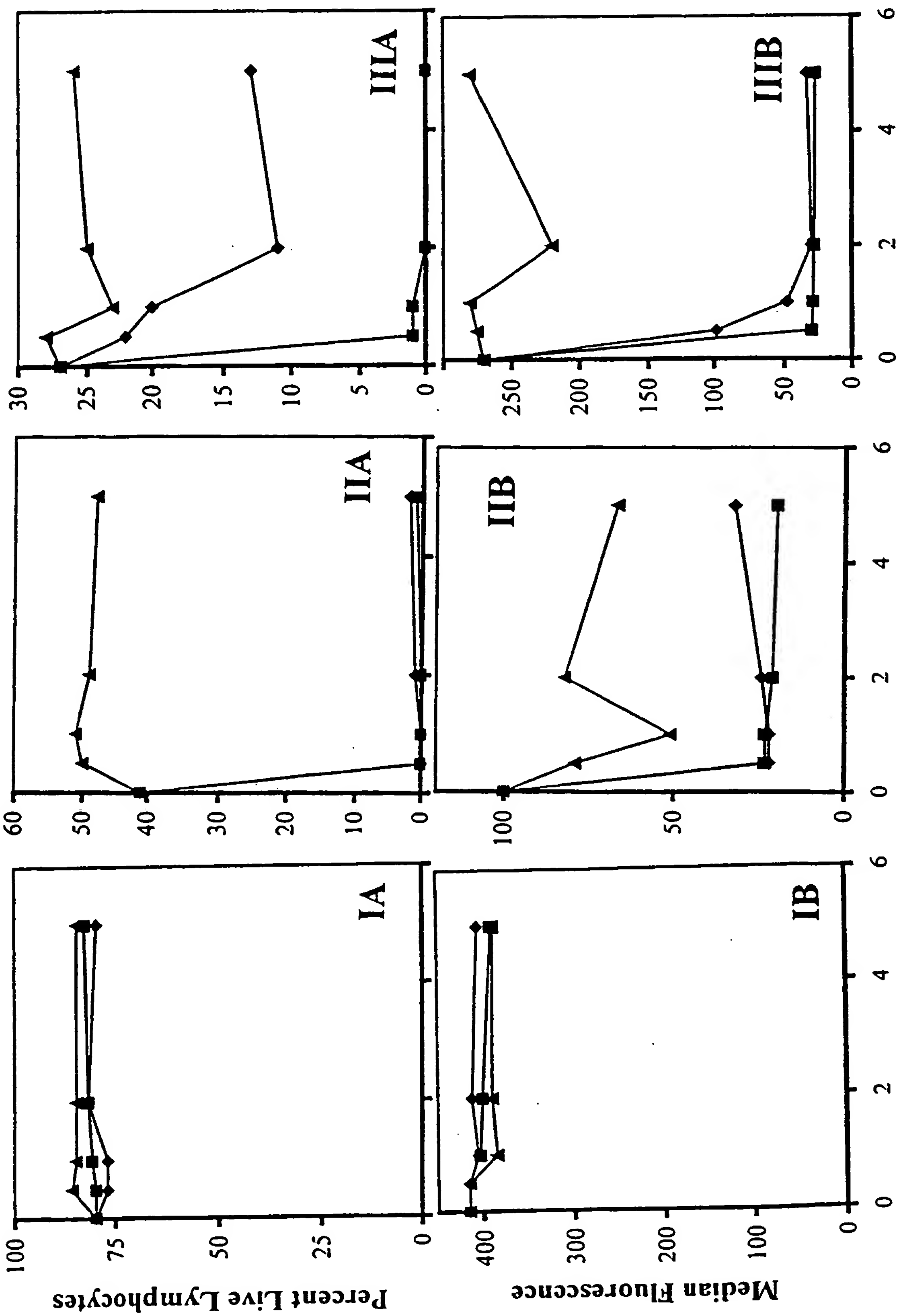


FIG. 2

FIG. 3A



Percent Live Lymphocytes
Median Fluorescence
IA
IB
IIA
IIB
IIIA
IIIB
100
75
50
25
0
400
300
200
100
0
60
50
40
30
20
10
0
100
50
0
30
25
20
15
10
5
0
250
200
150
100
50
0
2
4
6

FIG. 3B

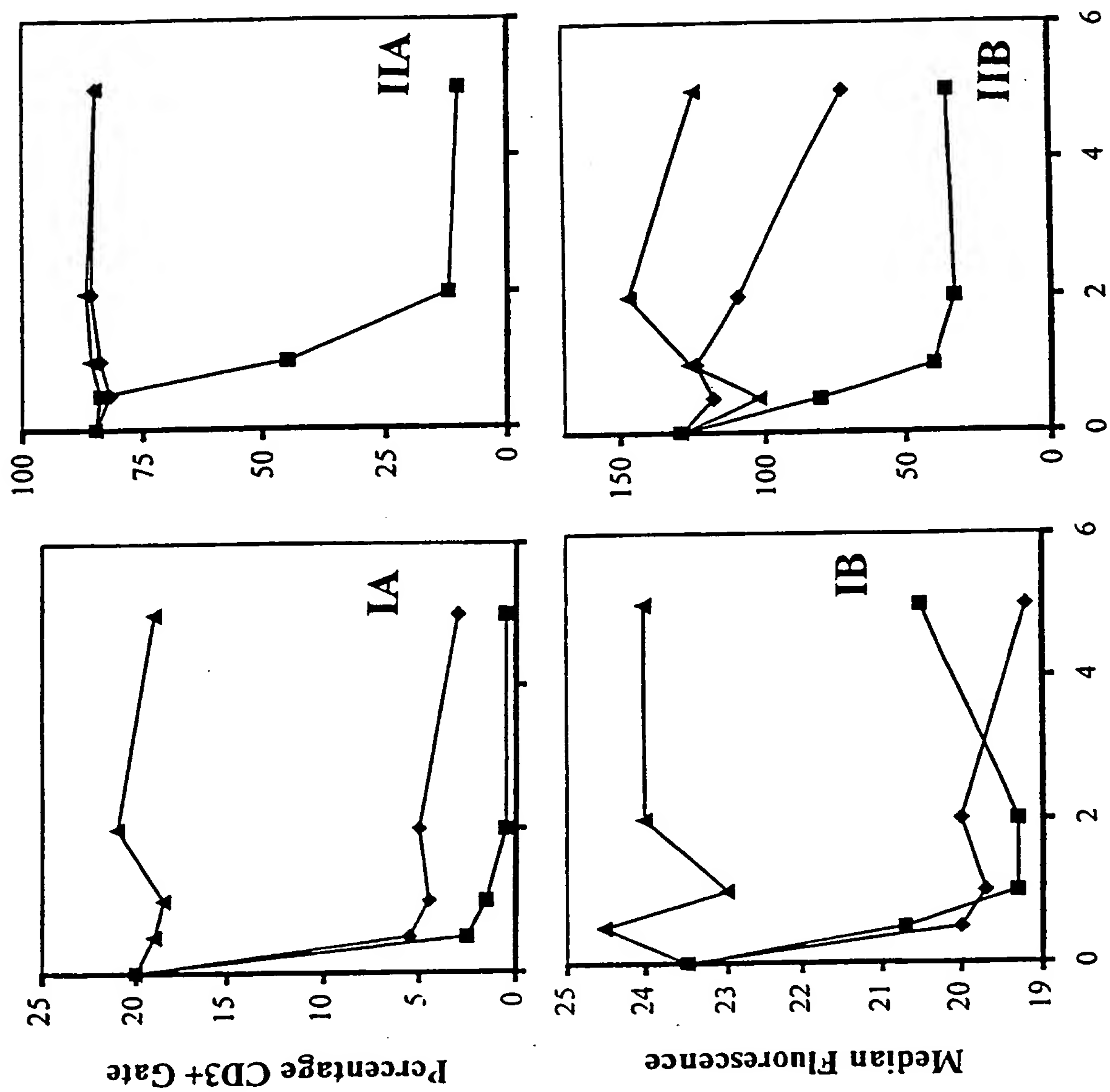

IA
IIA
IB
IIB
Percentage CD3+ Gate
Median Fluorescence
25
20
15
10
5
0
100
75
50
25
0
25
24
23
22
21
20
19
150
100
50
0
0
2
4
6
0
2
4
6

FIG. 3C

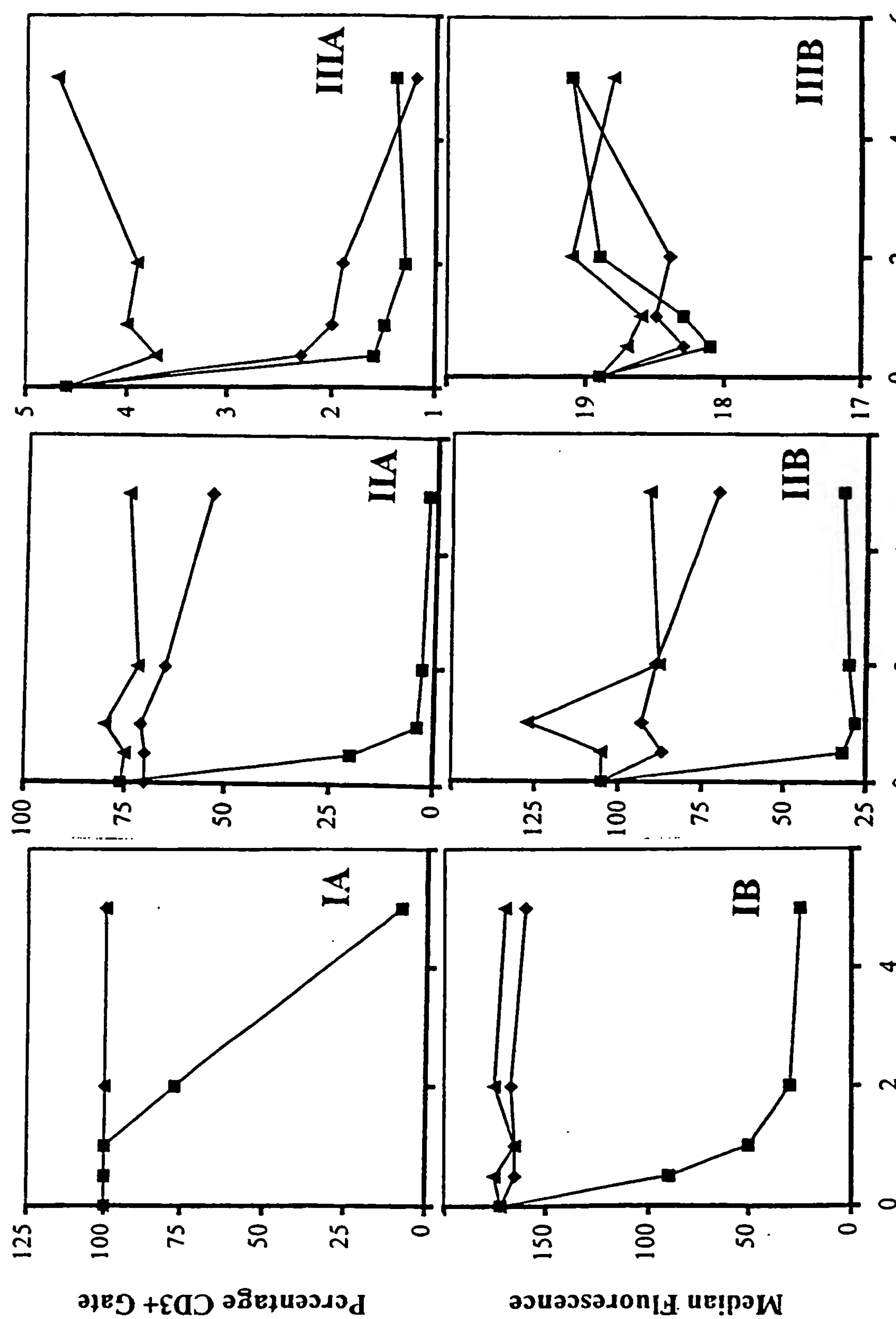

IA
IIA
IIIA
IB
IIB
IIIB
Percentage CD3+ Gate
Median Fluorescence

FIG. 4

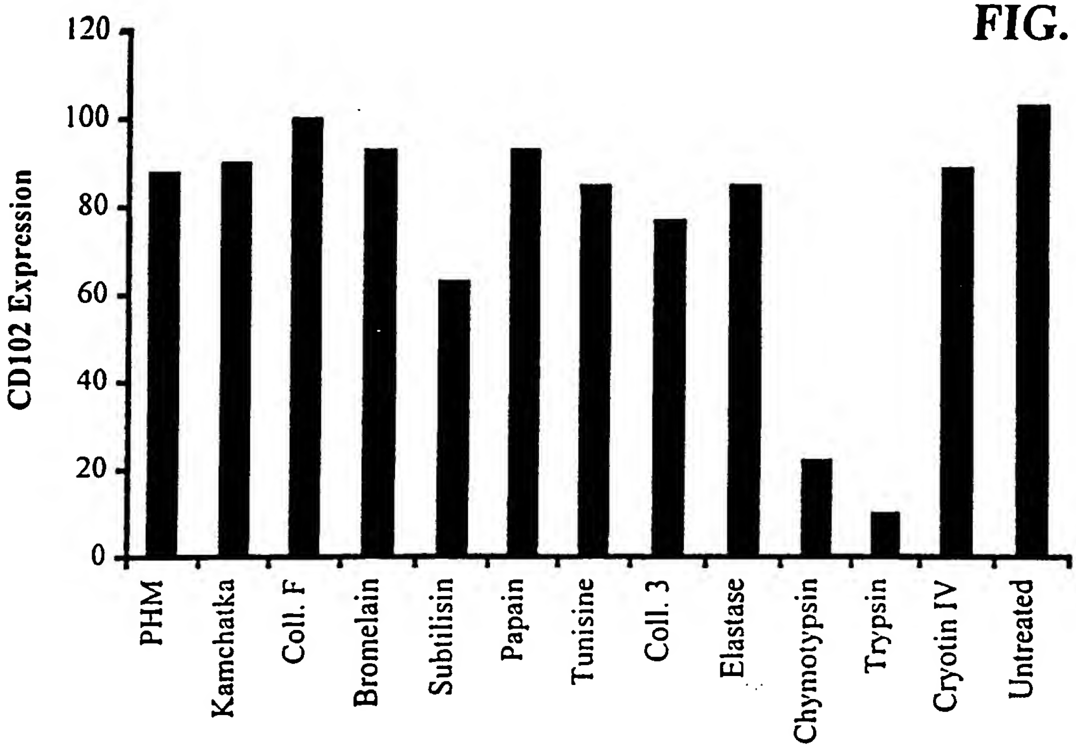


FIG. 4A

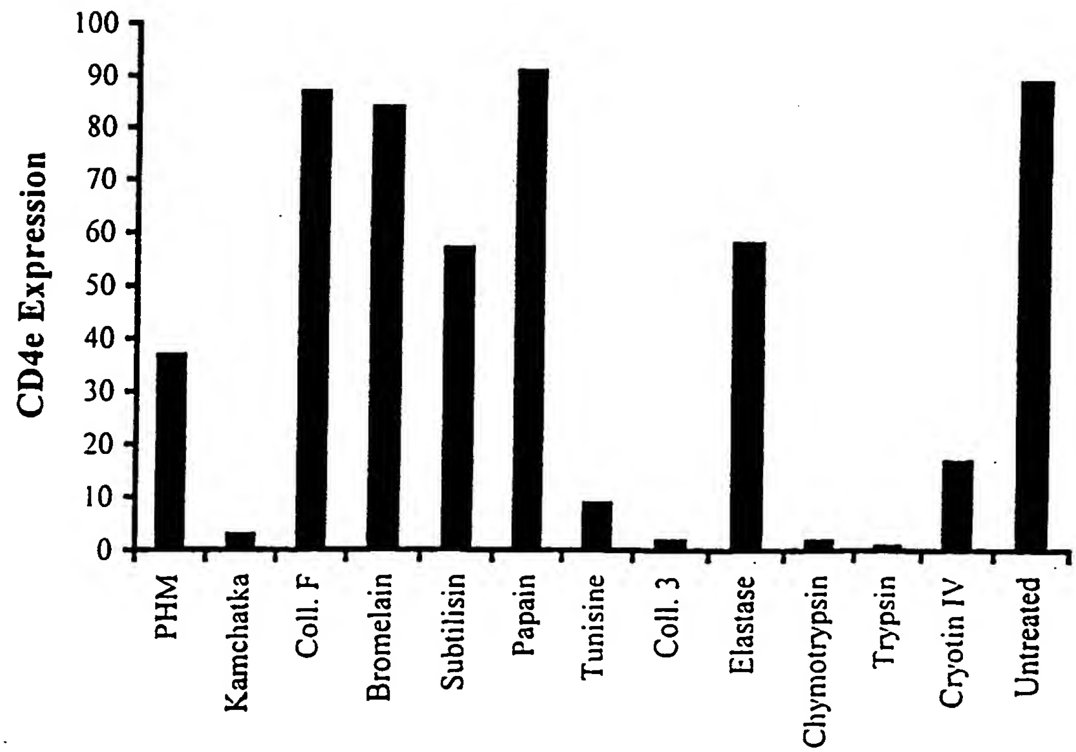
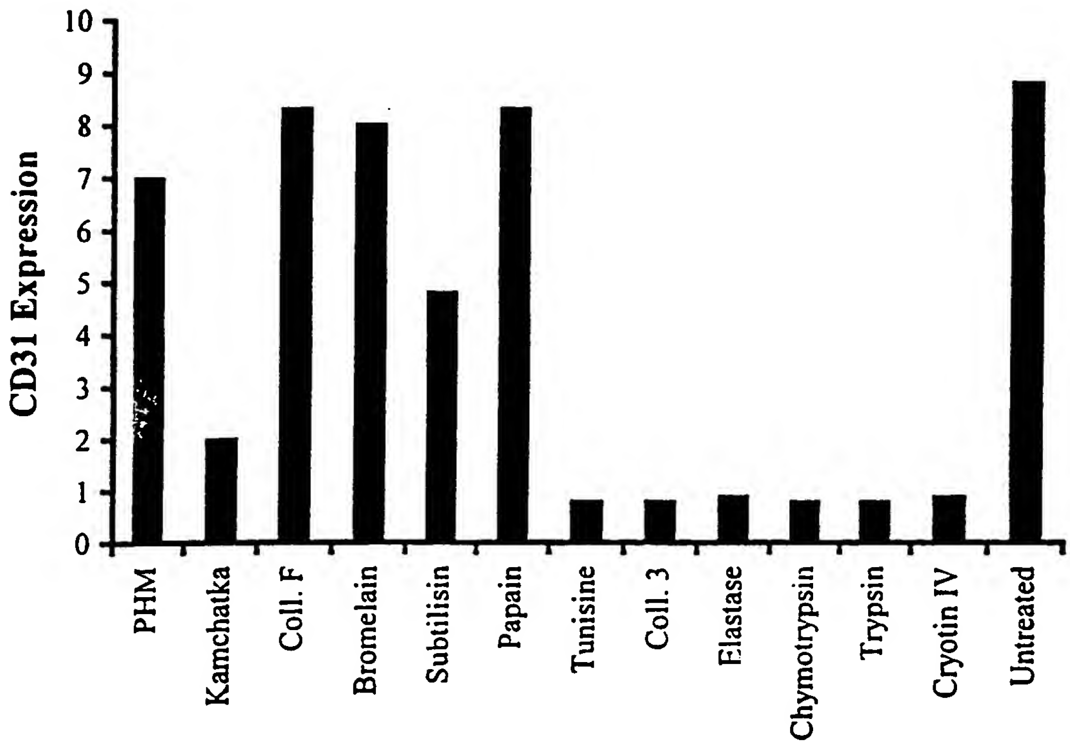


FIG. 4B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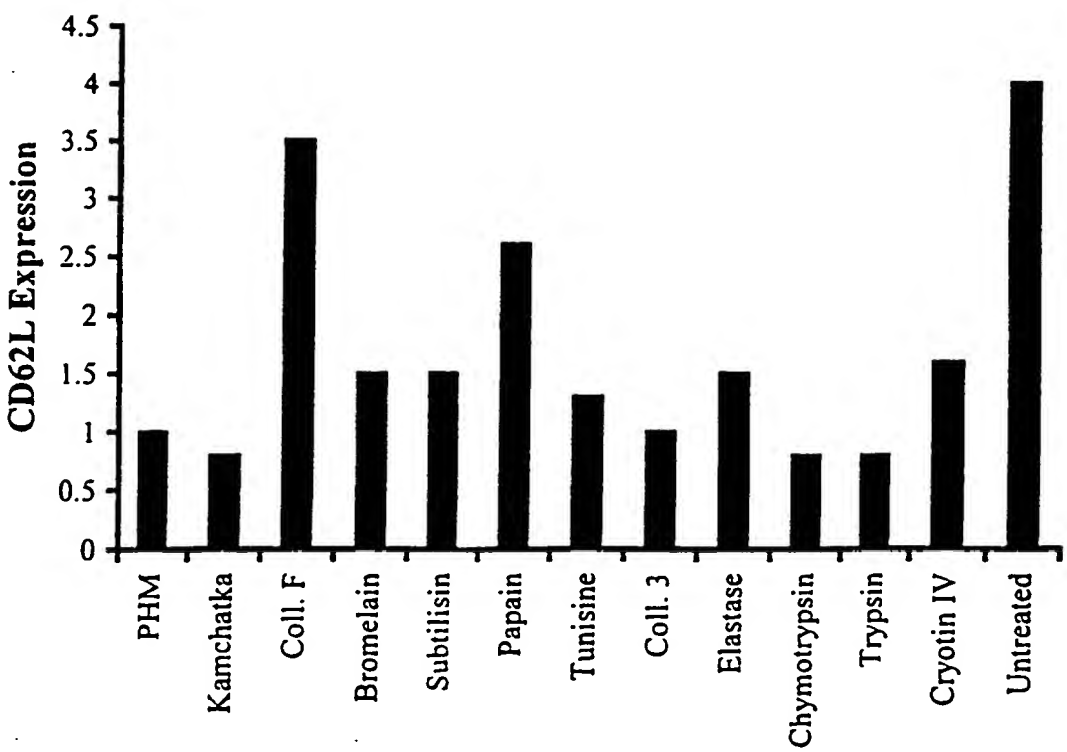
1
2
3
4
5
6
7
8
9
10
PHM
Kamchatka
Coll. F
Bromelain
Subtilisin
Papain
Tunisine
Coll. 3
Elastase
Chymotrypsin
Trypsin
Cryotin IV
Untreated

FIG. 4C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2
2.5
3
3.5
4
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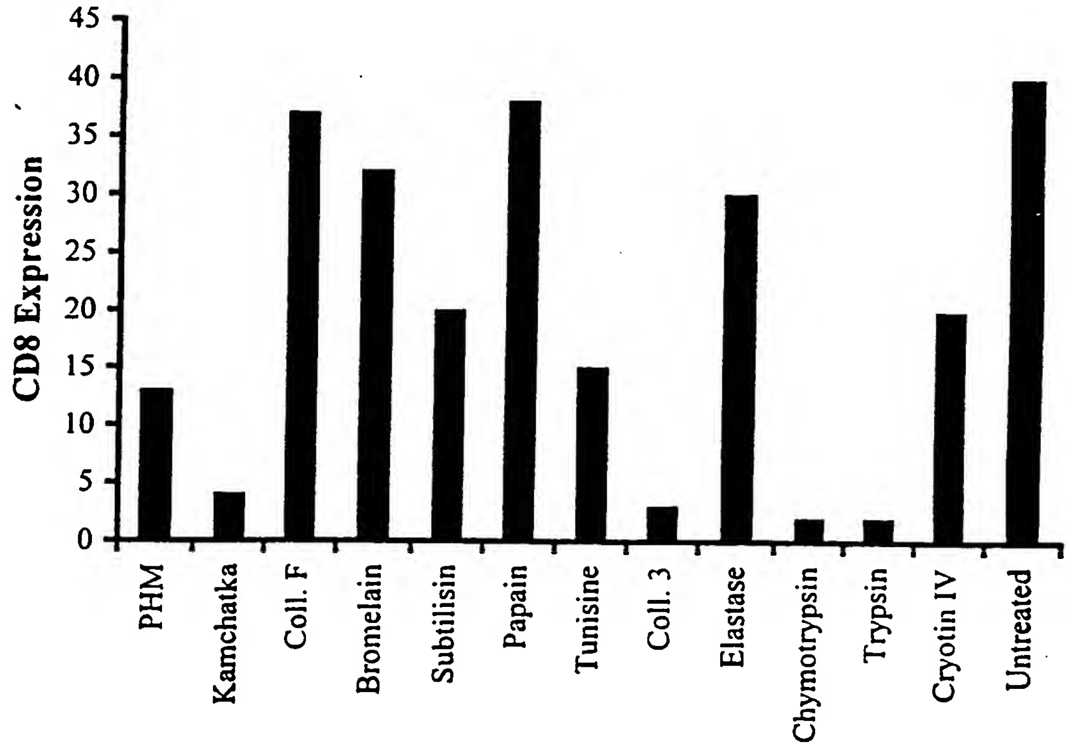
Kamchatka
Coll. F
Bromelain
Subtilisin
Papain
Tunisine
Coll. 3
Elastase
Chymotrypsin
Trypsin
Cryotin IV
Untreated

FIG. 4D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40
35
30
25
20
15
10
5
0
PHM
Kamchatka
Coll. F
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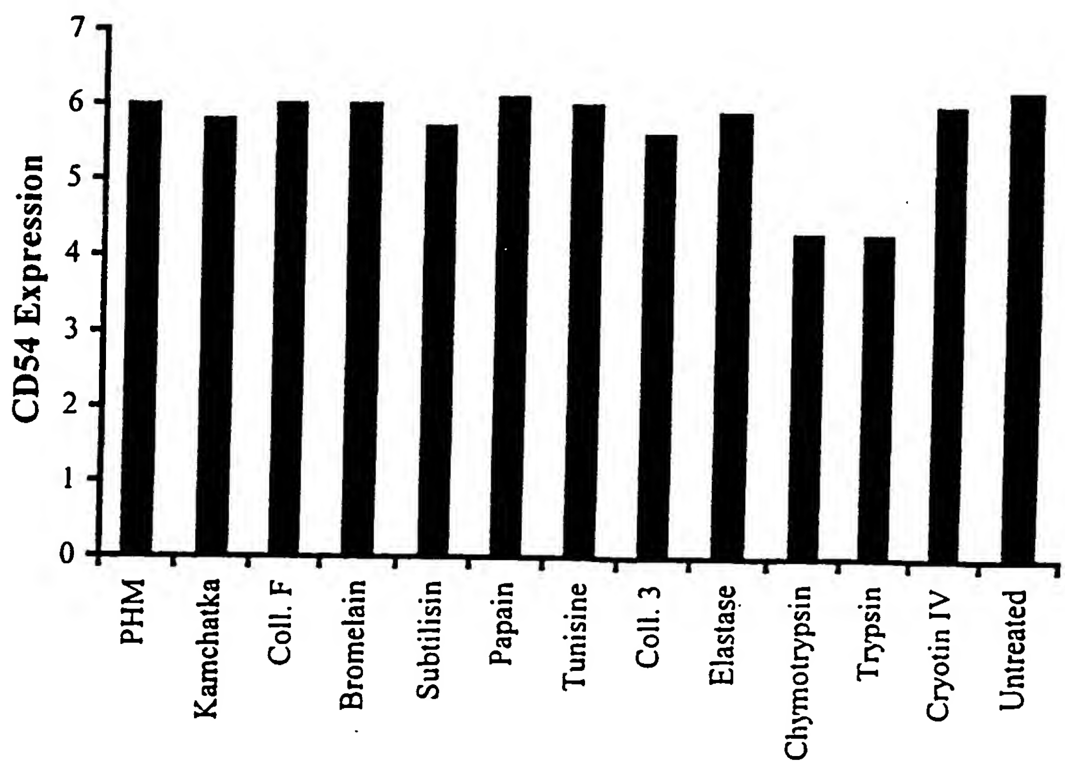
Subtilisin
Papain
Tunisine
Coll. 3
Elastase
Chymotrypsin
Trypsin
Cryotin IV
Untreated

FIG. 4E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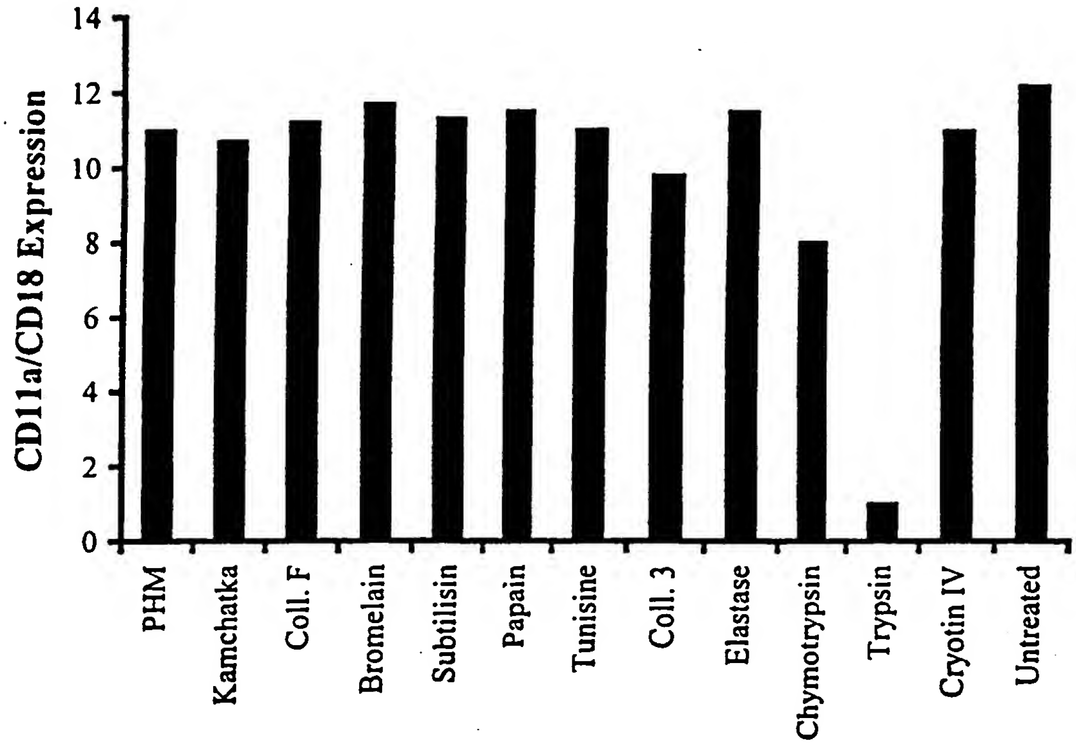
6
5
4
3
2
1
0
PHM
Kamchatka
Coll. F
Bromelain
Subtilisin
Papain
Tunisine
Coll. 3
Elastase
Chymotrypsin
Trypsin
Cryotin IV
Untreated

FIG. 4F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14
12
10
8
6
4
2
0
PHM
Kamchatka
Coll. F
Bromelain
Subtilisin
Papain
Tunisine
Coll. 3
Elastase
Chymotrypsin
Trypsin
Cryotin IV
Untreated

FIG. 4G

FIG. 5

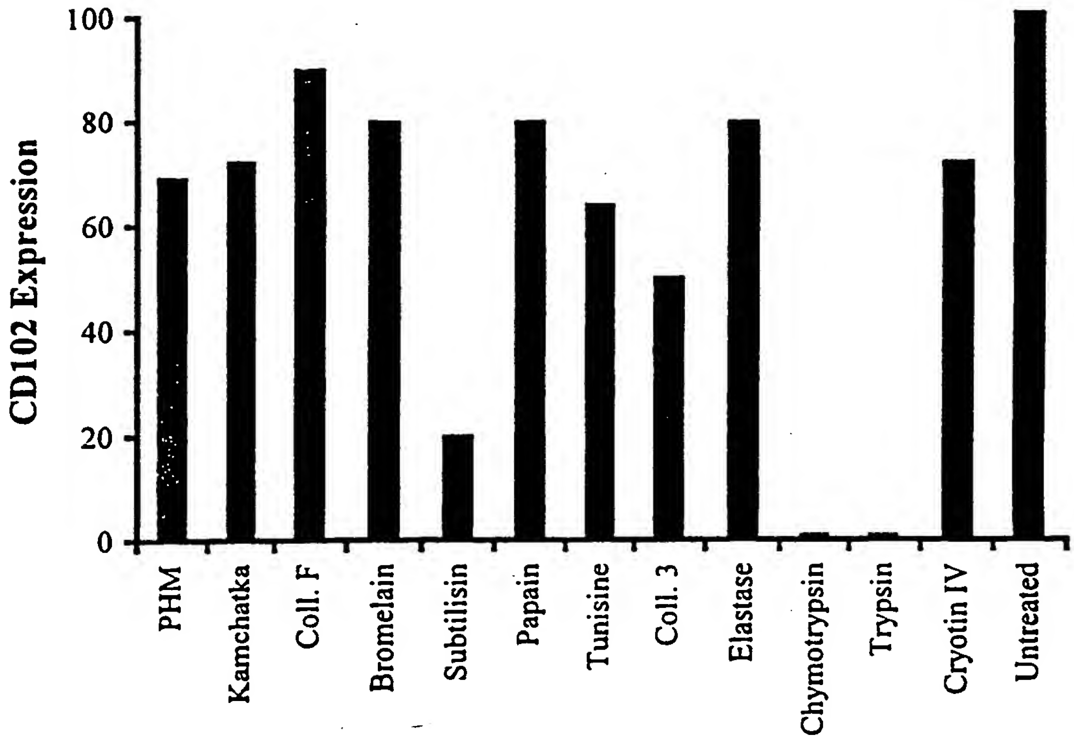


FIG. 5A

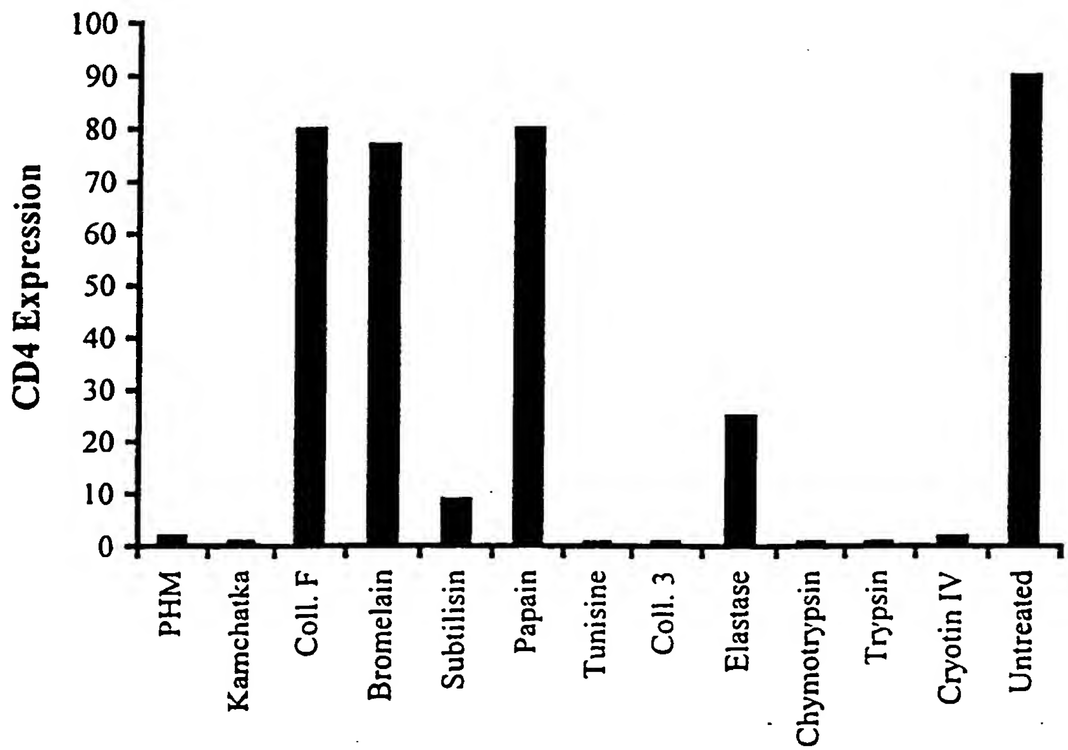
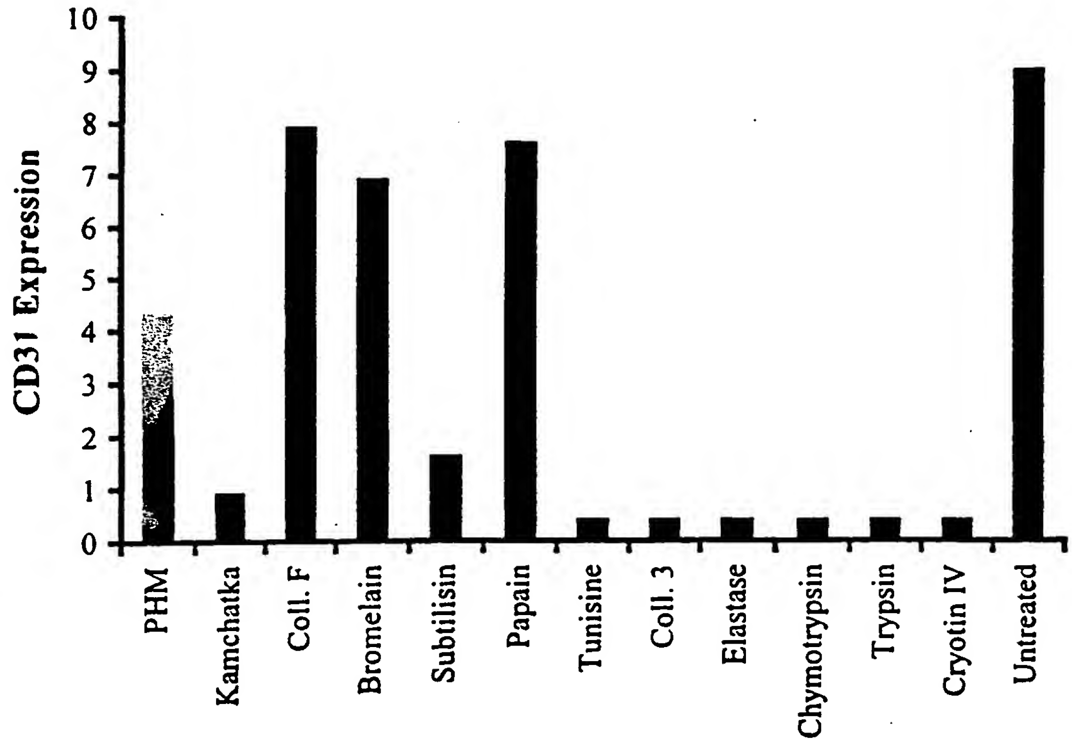


FIG. 5B



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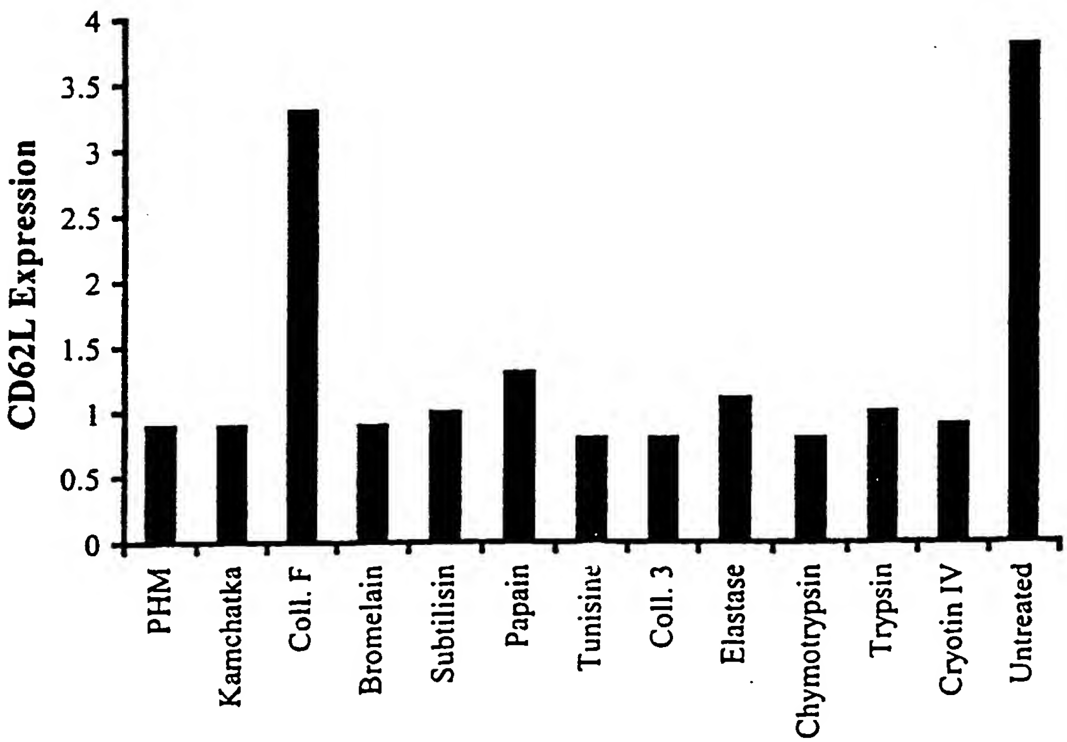
9
8
7
6
5
4
3
2
1
0
PHM
Kamchatka
Coll. F
Bromelain
Subtilisin
Papain
Tunisine
Coll. 3
Elastase
Chymotrypsin
Trypsin
Cryotin IV
Untreated

FIG. 5C



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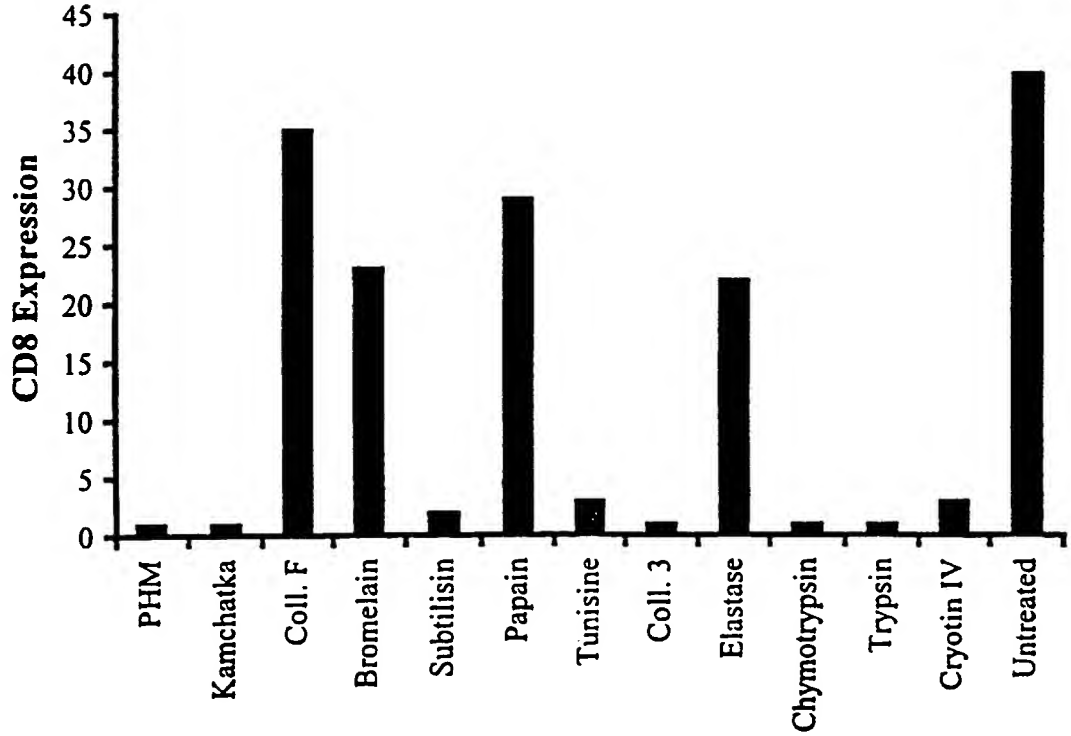
3.5
3
2.5
2
1.5
1
0.5
0
PHM
Kamchatka
Coll. F
Bromelain
Subtilisin
Papain
Tunisine
Coll. 3
Elastase
Chymotrypsin
Trypsin
Cryotin IV
Untreated

FIG. 5D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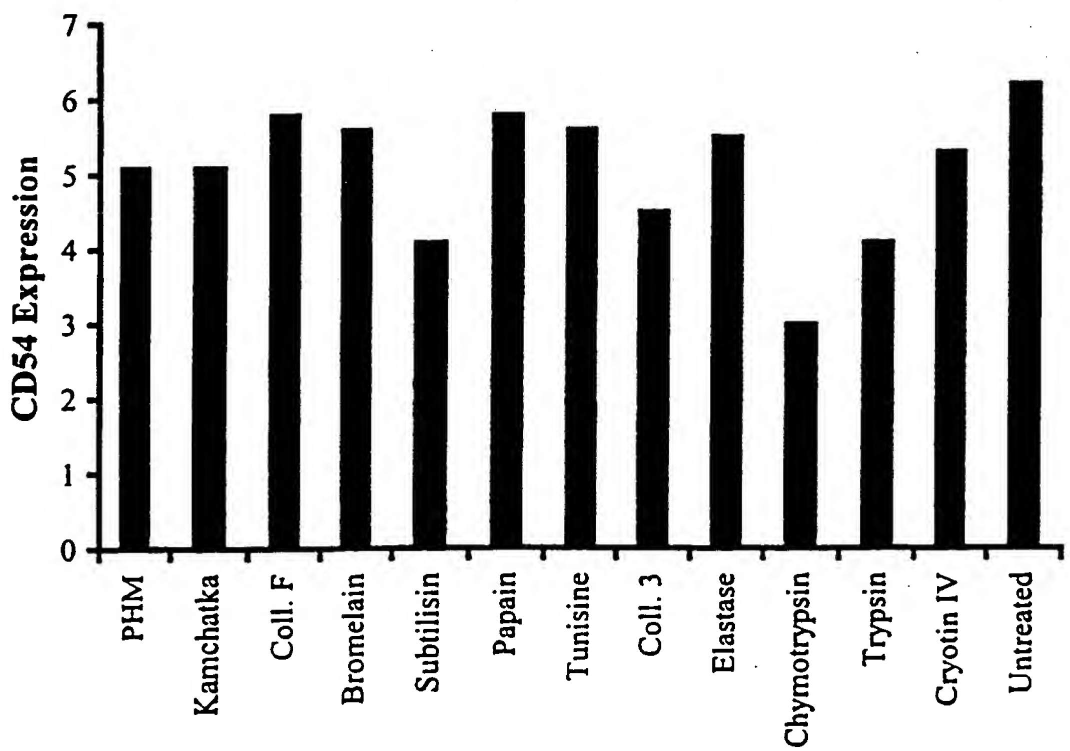
5
10
15
20
25
30
35
40
45
PHM
Kamchatka
Coll. F
Bromelain
Subtilisin
Papain
Tunisine
Coll. 3
Elastase
Chymotrypsin
Trypsin
Cryotin IV
Untreated

FIG. 5E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0
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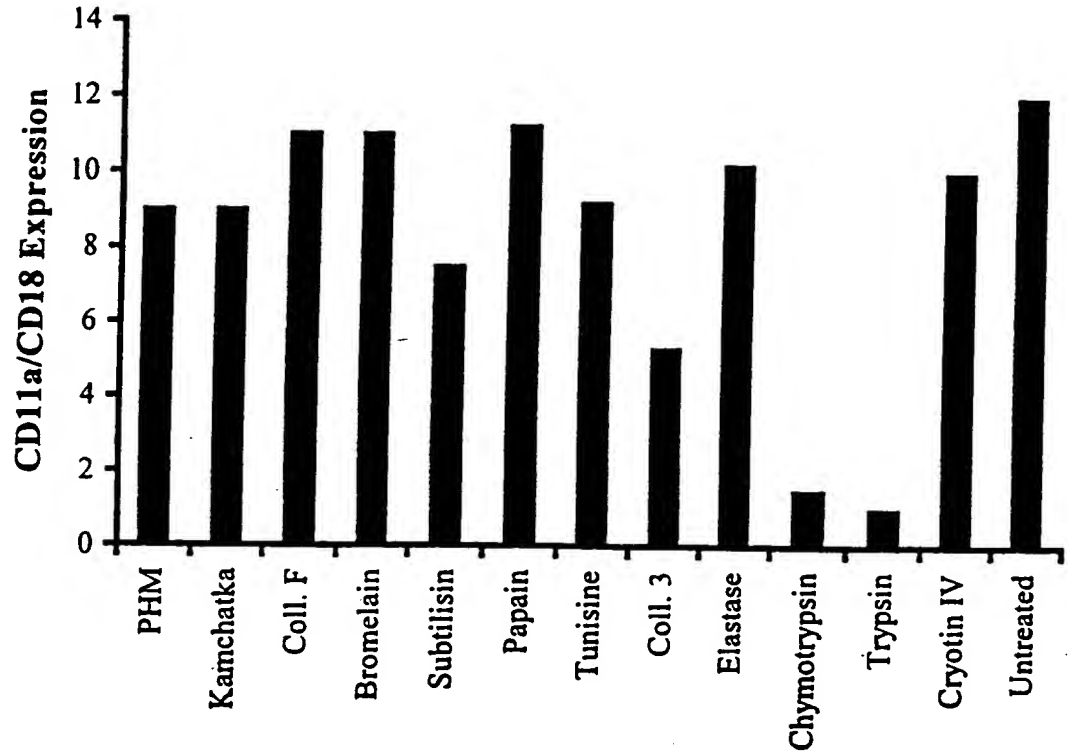
2
3
4
5
6
7
PHM
Kamchatka
Coll. F
Bromelain
Subtilisin
Papain
Tunisine
Coll. 3
Elastase
Chymotrypsin
Trypsin
Cryotin IV
Untreated

FIG. 5F



CD11a/CD18 Expression
14
12
10
8
6
4
2
0
PHM
Kamchatka
Coll. F
Bromelain
Subtilisin
Papain
Tunisine
Coll. 3
Elastase
Chymotrypsin
Trypsin
Cryotin IV
Untreated

FIG. 5G

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Yves St. Pierre

<120> Treatment and Prevention of Immune
Rejection Reactions

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            20   25

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20 25

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Ser Leu Gln Asp
20

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            20

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Ser Leu Gln Asp
            20

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<213> Salmon

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<213> Euphasia superba

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Gly Leu Phe Ile Asp Asp Met Tyr Phe
            20   25

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            20   25   30
Phe Arg Val Thr Lys Ser Ser Pro Val Gln Pro Pro Ala Val Arg Gly
        35   40   45
Thr Lys Ala Val Glu Asn Cys Gly Pro Val Ala Pro Arg Asn Lys Ile
    50   55   60

Val Gly Gly Met Glu Val Thr Pro His Ala Tyr Pro Trp Gln Val Gly
65 70 75 80
Leu Phe Ile Asp Asp Met Tyr Phe Cys Gly Gly Ser Ile Ile Ser Asp
85 90 95
Glu Trp Val Leu Thr Ala Ala His Cys Met Asp Gly Ala Gly Phe Val
100 105 110
Glu Val Val Met Gly Ala His Ser Ile His Asp Glu Thr Glu Ala Thr
115 120 125
Gln Val Arg Ala Thr Ser Thr Asp Phe Phe Thr His Glu Asn Trp Asn
130 135 140
Ser Phe Thr Leu Ser Asn Asp Leu Ala Leu Ile Lys Met Pro Ala Pro
145 150 155 160
Ile Glu Phe Asn Asp Val Ile Gln Pro Val Cys Leu Pro Thr Tyr Thr
165 170 175
Asp Ala Ser Asp Asp Phe Val Gly Glu Ser Val Thr Leu Thr Gly Trp
180 185 190
Gly Lys Pro Ser Asp Ser Ala Phe Gly Ile Ala Glu Gln Leu Arg Glu
195 200 205
Val Asp Val Thr Thr Ile Thr Thr Ala Asp Cys Gln Ala Tyr Tyr Gly
210 215 220
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245 250 255
Val Thr Gln Thr Arg Gly Ile Thr Ser Phe Gly Ser Ser Thr Gly Cys
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35 40 45
Thr Pro Glu Ile Gln Pro Val Cys Leu Pro Ser Tyr Thr Asp Ala Ala
50 55 60
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35 40 45
Val Gln Pro Pro Ala Val Arg Gly Thr Lys Ala Val Glu Asn Cys Gly
50 55 60
Pro Val Ala Pro Lys Asn Lys Ile Val Gly Gly Gln Glu Val Thr Pro
65 70 75 80
His Ala Tyr Pro Trp Gln Val Gly Leu Phe Ile Asp Asp Met Tyr Phe
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Cys Gly Gly Ser Ile Ile Ser Glu Asp Trp Val Leu Thr Ala Ala His
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Cys Val Asp Gly Ala Gly Phe Val Glu Val Val Met Gly Ala His Ser
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Ile His Asp Asp Thr Glu Ala Ser Arg Ile Ser Ala Thr Ser Thr Asp
130 135 140
Phe Phe Thr His Glu Asn Trp Asn Ser Phe Thr Leu Thr Asn Asp Leu
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35 40 45
Val Gln Pro Pro Ala Val Arg Gly Thr Lys Ala Val Glu Asn Cys Gly
50 55 60
Pro Val Ala Pro Lys Asn Lys Ile Val Gly Gly Gln Glu Val Thr Pro
65 70 75 80
His Ala Tyr Pro Trp Gln Val Gly Leu Phe Ile Asp Asp Met Tyr Phe
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Phe Gly Gly Ser Ile Ile Ser Glu Asp Trp Val Val Thr Ala Arg His
100 105 110
Cys Met Asp Gly Arg Gly Phe Val Glu Val Val Met Gly Ala His Ser
115 120 125
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Phe Phe Thr His Glu Asn Trp Asn Ser Phe Thr Leu Thr Asn Asp Leu

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Pro Val

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Glu Ser Val Thr Leu Thr Gly Trp Gly Lys Pro Ser Asp Ser Ala Phe
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Tyr Thr Asp Ser Asp Asp Asp Phe Val Gly Glu Ser Val Thr Leu Thr
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/30818

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 38/43, 38/46, 39/395
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.1, 94.6, 130.1., 133.1, 141.1, 143.1, 144.1, 153.1, 154.1, 173.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, BIOSIS, CA, EMBASE, MEDLINE, USPAT
search terms: hydrolase, krill, phm protease, trypsin, papain, cd4, cd8, cd25, cd28, ctla-4, cd40, cd40 ligand, cd80, b7

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,747,037 A (NOELLE ET AL.) 05 May 1998, see entire document.	1-19
Y	US 5,747,034 A (DE BOER ET AL.) 05 May 1998, see entire document.	1-19
Y	US 5,756,096 A (NEWMAN ET AL.) 26 May 1998, see entire document.	1-19
Y	WO 98/38291 A1 (CORTECS LIMITED) 03 September 1998, see entire document.	1-19

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 MARCH 2000	26 APR 2000

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	PHILLIP GAMBEL Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KRADIN et al. Antigen-independent Binding of T-Cells by Dendritic Cells and Alveolar Macrophages in the Rat. AM. REV. RESPIR. DIS. 1989, Volume 139, pages 207-211, see entire document.	1-19
Y	MANNHALTER et al. Modulation of Antigen-Induced T Cell Proliferation by α_2M-Trypsin Complexes. J. Immunol. 15 April 1996, Volume 136, No. 8, pages 2792-2799, see entire document.	1-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/30818

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/94.1, 94.6, 130.1., 133.1, 141.1, 143.1, 144.1, 153.1, 154.1, 173.1